THE NEW IASLC/ATS/ERS CLASSIFICATION WITH CLINICOPATHOLOGICAL FEATURES OF PRIMARY LUNG ADENOCARCINOMAS AND CORRELATION WITH EGFR MUTATIONAL STATUS.

A DISSERTATION SUBMITTED IN PART FULFILMENT OF THE REQUIREMENTS FOR THE M.D. DEGREE BRANCH III (PATHOLOGY) EXAMINATION OF THE TAMIL NADU DR. M.G.R. MEDICAL UNIVERSITY CHENNAI TO BE HELD IN APRIL 2015

DECLARATION

This is to declare that this dissertation titled**"** ***THE NEW IASLC/ATS/ERS CLASSIFICATION WITH CLINICO-PATHOLOGICAL FEATURES OF PRIMARY LUNG ADENOCARCINOMAS AND CORRELATION WITH EGFR MUTATIONAL STATUS****"** is my original work, under the guidance and supervision of my guide, Dr (Prof). Anila Korula (MBBS, MD) in part fulfilment of the requirement for the M.D. Branch III (Pathology) Degree Examination of the Tamil Nadu Dr. M.G.R. Medical University, Chennai, to be held in April 2015. I have independently reviewed the literature, standardized the data collection methodology and carried out the evaluation towards completion of the thesis.

Dr. Ravi Priyanka Yogendra,

M.D Pathology Postgraduate,

Department of Pathology,

Christian Medical College,

Vellore.

CERTIFICATE

This is to certify that this dissertation titled ***"THENEW IASLC/ATS/ERS CLASSIFICATION WITH CLINICO-PATHOLOGICAL FEATURES OF PRIMARY LUNG ADENOCARCINOMAS AND CORRELATION WITH EGFR MUTATIONAL STATUS"*** is a bonafide work done by Dr.Ravi Priyanka Yogendra, in part fulfilment of rules and regulations for the M.D. Branch III (Pathology) Degree examination of The Tamil Nadu Dr. M.G.R. Medical University, to be held in April 2015.

HEAD OF DEPARTMENT

Dr. Banumathi Ramakrishna,
MBBS, MD,MAMS.
Professor & Head,
Department of Pathology,
Christian Medical College,
Vellore.

PRINCIPAL:

Dr. Alfred Job Daniel
D(ortho), MS(ortho), DNB(ortho)
Professor,
Dept. of Orthopaedics,
Christian Medical College,
Vellore.

CERTIFICATE

This is to certify that this dissertation titled ***"THE NEW IASLC/ATS/ERS CLASSIFICATION WITH CLINICO-PATHOLOGICAL FEATURES OF PRIMARY LUNG ADENOCARCINOMAS AND CORRELATION WITH EGFR MUTATIONAL STATUS"*** is a bonafide work done by Dr.Ravi Priyanka Yogendra, under my guidance, in part fulfilment of the requirement for the M.D. Branch III (Pathology) Degree Examination of the Tamil Nadu Dr. M.G.R. Medical University, Chennai, to be held in April 2015.The candidate has independently reviewed the literature, standardized the data collection methodology and carried out the evaluation towards completion of the thesis.

Dr. Anila Korula, MBBS, MD,

Professor,

Department of Pathology,

Christian Medical College,

Vellore.

CONTENTS

LIST OF TABLES.

LIST OF FIGURES

ABBREVIATIONS.

AIS	Adenocarcinoma in situ
BAC	Bronchioloalveolar carcinoma.
EGFR	Epidermal growth factor receptor.
EML4-ALK	Human Echinoderm Microtubule Associated Protein-Like- 4 Anaplastic Lymphoma Kinase
IASLC/ATS/ERS	International Association for the Study of Lung Cancer/ American Thoracic Society/European Respiratory Society Classification.
KRAS	V-Ki-ras2Kirsten Rat Sarcoma Viral Oncogene Homolog
MIA	Minimally invasive adenocarcinoma.
Nap- A	Napsin A.
NSCLC-NOS	Non small cell lung carcinoma- not otherwise specified.
TKI	Tyrosine kinase inhibitor.
WHO	World Health Organization
dNTPs	Deoxyribonucleotide triphosphate
FFPE	Formalin fixed paraffin embedded
PCR	Polymerase chain reaction.
TBS	TRIS Buffered Saline
LCNEC	Large cell neuroendocrine carcinoma.

TITLE: THE NEW IASLC/ATS / ERS CLASSIFICATION WITH CLINICO-PATHOLOGICAL FEATURES OF PRIMARY LUNG ADENOCARCINOMAS AND CORRELATION WITH EGFR MUTATIONAL STATUS.

Department : General Pathology.

Name of the candidate : Dr. Ravi Priyanka Yogendra.

Degree and Subject : M.D Pathology.

Name of the Guide : Dr. Anila Korula.

Aim of the study: To do a detailed histopathological study of all Primary Lung Adenocarcinomas using the New IASLC/ATS/ERS classification, biopsied in our institution from June 2011- December 2012 and correlate this data with EGFR mutational status .

Objectives:

1. To categorize and study the detailed histopathological features of all Primary Lung Adenocarcinomas using the 2011 New International Classification (IASLC/ATS/ERS) classification for lung adenocarcinomas .

2. To correlate these histopathological features with data available on EGFR mutational status performed by PCR Sequencing.

3. To further sub-classify these adenocarcinomas that is predominantly associated with EGFR mutations.

4. To correlate the above with parameters such as gender, age, smoking, status and stage of disease.

Clinical and statistical methods:

Two hundred and seventy four cases were categorized using the IASLC/ETS/ARS classification and correlated with EGFR exon sequences performed in a subset of 120 cases using PCR Sequencing. TTF-1 immunohistochemistry was performed in majority of cases. Demographic details including age, gender, smoking status and geographical location were obtained from the electronic computerized medical records. Radiological findings were obtained from the CT scan results from the Picture Archiving and Communication System.

Data was analyzed using the SPSS software. Descriptive statistics for continuous data were analyzed using mean with Standard deviation or median with inter-quartile range. Categorical data was described using frequencies and percentages. Histopathological subtypes, cell type and clinico-pathological features with EGFR mutational analysis were associated using Fischer's exact test and Chi square test.

Results: The predominant biopsy categories of adenocarcinoma were, invasive acinar (61%), solid with mucin production (23%), mucinous (19%) and lepidic (4%). EGFR mutations were detected in 40.9% cases, mainly with lepidic (60%), papillary (44.4%), acinar (42.7%) and solid variants (41.7%) and cuboidal cell type (P=0.013) and infrequent with mucinous subtype (P=0.015).The commonest form of mutation involved exon 19 in 69.4% cases. Majority were TTF-1 positive (81%, P=0.007), mainly associated with lepidic and papillary subtypes. The polygonal cell type was frequently noted among grade III tumours, in stage IV and smokers. The solid variant was relatively frequent in smokers and was poorly differentiated. Thus, in this study we conclude that the New

IASLC/ATS/ERS classification confirms the positive statistical association of the predominant subtype of invasive adenocarcinoma and the cell type with EGFR mutations and TTF-1 reactivity.

Keywords: New IASLC/ATS/ERS classification, Adenocarcinoma, Lung, Epidermal growth factor receptor, TTF-1.

Introduction

Lung cancer continues to be a leading cause of mortality globally. Worldwide it comprises 17% of the new cancer cases and 23% of the cancer deaths in men. The mortality burden for females in developing countries is similar to cervical cancer in women, approximately 11% of the female cancer deaths(1). The 2008 GLOBOCAN reported that India had around 47,010 new cases of lung malignancy in males and around 11,557 new cases of lung malignancy in females (2) In India, it is the most frequently diagnosed malignancy in males and the 4th most common together in both genders.

Lung cancers are broadly classified based on histological features and response to conventional therapies as small cell lung cancer and non small cell lung cancer (NSCLC), latter being the commonest (3). Non small cell lung carcinoma includes three subtypes mainly, squamous cell carcinoma, large cell carcinoma and adenocarcinoma and these can be further divided into various subtypes or variants (4) , adenocarcinoma being the commonest histological subtype of NSCLC (5)(6). Smoking is an established major risk factor identified in all types of lung carcinoma, most commonly associated with male smokers and squamous cell carcinoma being the histological predominant subtype (7). Adenocarcinoma is the most common subtype in non smokers and female gender, with increasing incidence rates with time.(7)

Until recently, NSCLC was treated as a single disease with "one size fits all' therapeutic approach with conventional chemotherapeutic effects. Each subtype was found to display distinct patterns of genomic alterations with different response rates, toxicity and progression free survival with targeted drugs like bevacizumab, pemetrexed and tyrosine kinase inhibitors. (6)

Lung cancers are the most histologically variable and display heterogeneity between cases and within individual tumours, which is important for pathologists reporting this tumour type. Small biopsies samples which form the diagnosticbulk may not be representative of the tumour.(8). Inspite of this heterogeneity, histology is still regarded recognized as an important factor in treatment selection (6)(9)(10).

The world health organization (WHO) classification in 1999, introduced the adenocarcinoma of the mixed subtype category, which was maintained in 2004 WHO classification of lung tumours, 2004(11)(12). The shortcoming of WHO classification, 1999/2004 was that the mixed subtype of adenocarcinoma comprised 94% of cases in one of the series(13). Inspite of being pathologically accurate, it is of limited clinical utility as most adenocarcinomas fell into this category despite varied outcomes .(14)(15). The micropapillary carcinoma was not included as an independent subtype, which has emerged as a important poor subtype of lung adenocarcinoma adenocarcinoma in early-stage tumours (16,17). Besides these major flaws both mucinous and non- tumours were clubbed under the category of bronchioloalveolar carcinoma (BAC) although they have different clinical,

radiological, pathologic and molecular features. In resection specimens, AISand MIA were not separately classified inspite of 100% 5 year disease free survival after complete resection.(18).

Due to the rapidly evolving advances in the field of lung adenocarcinoma, there was an emergent need for improvement in histological categorization of lung adenocarcinomas according to prognosis as compared to the previous classifications. To address various issues, in 2008 an multidisciplinary panel sponsored by the International Association for the study of Lung Cancer (IASLC), American Thoracic Society (ATS) and European Respiratory Society (ERS), proposed the new IASLC/ATS/ERS International Lung Adenocarcinoma Classification, published in 2011(19). This classification highlights several paradigm shifts to overcome the pitfalls of the WHO 2004 classification, and to precisely diagnose and sub-classify lung adenocarcinomas, in view of the established relationship between histologic classification and predictive molecular markers.

Thus, with the guidelines of the current IASLC/ETS/ARS classification it is possible to categorize and prognosticate lung cancers as per particular subtype. In this era of personalized treatment for lungcarcinoma based on the histology (adenocarcinoma versus squamous) and molecular status (i.e., epidermal growth factor receptor [EGFR] mutation and anaplastic lymphoma kinase [ALK] rearrangement in adenocarcinoma), the pathologist's role is vital. There are specific treatments for patients depending on the histology and the molecular status of tumour.

Thus, we categorized and performed a detailed histopathological analysis of lung adenocarcinomas including poorly differentiated carcinomas using the current IASLC/ETS/ARS classification in an Indian cohort.

AIMS & OBJECTIVES

Aim of the study: To do a detailed histopathological study of all Primary Lung Adenocarcinomas using the new International classification, biopsied in our institution from June 2011- December 2012 and correlate this data with EGFR mutational status.

Objectives:

1. To categorize and study the detailed histopathological features of all Primary Lung Adenocarcinomas including Poorly Differentiated Carcinomas using the 2011 New International Classification (IASLC/ATS/ERS) classification for lung adenocarcinomas .

2. To correlate these histopathological features with data available on EGFR mutational status performed by PCR Sequencing to further sub-classify these adenocarcinomas that is predominantly associated with EGFR mutations.

3. To correlate the above with parameters such as gender, age, smoking status and stage of disease.

REVIEW OF LITERATURE.

Lung carcinomas display heterogeneity between cases and within individual tumours, with profound implications for exact histopathological categorization of these tumours. Approximately 70% of lung carcinomas are unresectable as these patients present in end stage of disease, hence small biopsies which form the diagnostic bulk might be unrepresentativeof the tumourarchitecture. (8)(20)Despite this morphological heterogeneity, histology is still regarded recognized as an important factor in treatment selection. (6)(9)(10)

Histogenetic origin of Lung Adenocarcinoma

Lung tissue from where lung carcinomas arise are dividedanatomically as the air conducting system and lung parenchyma in the periphery, associated with gaseous exchange. TTF-1 is expressed in the periphery in small bronchioles and alveoli. The peripheral bronchioloalveolar compartment comprisingof terminal bronchioles, alveoli and alveolar ducts are lined by 2 potential cells of tumour origin, the type II pneumocytes and Clara cells, together comprising terminal respiratory unit (TRU) that give rise to tumours that express TTF-1. These tumours manifest as ground glass nodules on CT.

The bronchi contain the bronchial basal cells and the mucous cells, the potential progenitor tumour cells, giving rise to tumours that are TTF-1

negative and show a solid appearance on imaging.. Thus, the expression profile of lung adenocarcinomas includes that of TRU, and the non TRU adenocarcinomas, with distinct histogenetic origins. (19,21)

In 2004, the World Health Organization (WHO) classified lung adenocarcinomas as follows (12)

2004 WHO HISTOLOGICAL CLASSIFICATION OF LUNG ADENOCARCINOMA

Bronchioloalveolar carcinomas

- Mucinous type
- Non mucinous type
- Mixed non-mucinous & mucinous or indeterminate type

Acinar adenocarcinomas.

Papillary adenocarcinomas.

Solid adenocarcinomas

Variant

- Fetal adenocarcinomas.
- Mucinous cystadenocarcinomas.
- Colloid carcinomas.
- Signet ring adenocarcinomas
- Clear cell adenocarcinomas.

The (2004) WHO classification categorized bronchioloalveolar carcinomas (BAC) into two subtypes namely mucinous and non-mucinous

which have been reclassified by the New International Classification as mucinous/ non mucinous Lepidic predominant adenocarcinomas respectively(18).

Lepidic-predominant adenocarcinoma, previously known as bronchioloalveolar carcinoma (BAC) typically consists of bland pneumocytes (type II pneumocytes) that grow along surface of alveolar walls. The invasive adenocarcinoma component should be present in atleast 1 focus, >5mm in greatest dimension. The invasive component is defined as the presence of histologic subtypes apart from than a lepidic pattern and / presence of myofibroblastic stroma associated with invasive tumour cells. A diagnosis of lepidic-adenocarcioma is made in cases of MIA if the cancer cells demonstrate lymphovascular invasion, invade pleura or (ii) presence of tumour necrosis(18)

The non mucinous lepidic predominant typically shows Clara cell and/or type II cell differentiation. The Clara cells are columnar with cytoplasmic snouts and havepaleeosinophilic cytoplasm. Nuclei might be apical in location. Type II cells are dome-shaped or cuboidal with fine cytoplasmic vacuoles or clear to foamy cytoplasm.(12). On immunohistochemistry these tumours are positive for TTF-I and CK7 and negative for CK 20. They probably show EGFR mutation and only occasionally associated with KRAS mutations.(18)

Invasive mucinous adenocarcinoma previously addressed as mucinous BAC with distinctive histological features. The cells have columnar

or goblet cell morphology with abundant cytoplasmic mucin with minimal or absent nuclear atypia. The alveoli also contain mucin. These tumours may show heterogeneity of lepidic, acinar, micropapillary, papillary, and solid growth as nonmucinous tumours. They have to be differentiated from adenocarcinomas producing mucin but without the goblet cell or columnar morphology, that resemble and have been classified as mucinous BAC. The diagnosis of such tumours are suffixed with the term ‘‘with mucin production’’ or ‘‘with mucinous features(18)

On immunohistochemistry they are positive for CK7, CK20 and negative for TTF-I. The separation of these tumours is important because they have the most robust histological- molecular correlation with KRAS mutations and EGFR mutations. Metastatic mucinous adenocarcinomas from sites such as the pancreas and ovary can appear morphologically identical to pulmonary invasive mucinous adenocarcinoma(22)

Pancreatic mucinous adenocarcinomas are likely to express cytokeratin (CK) 20 and mucin 2 (MUC2). Metastatic colorectal adenocarcinomas often express caudal-related homeobox 2 (CDX-2) and CK20 with lack of CK7 (18) The invasive mucinous adenocarcinomas apart from having a different histological appearance from the non mucinous BAC, also presents frequently with multiple nodules, lobar consolidation and or bilateral lung involvement with frequent KRAS mutations as opposed to EGFR mutations.(19)

Tumours with mixed mucinous and non-mucinous features are rare, if so, the percentages of invasive mucinous adenocarcinoma have to be

documented. Tumours with atleast 10% component of each mucinous and non- mucinous are categorized as mixed invasive mucinous and non mucinous adenocarcinomas.

Acinar adenocarcinoma has amajority of round /oval glandular structures with a central luminal space, surrounded by these tumour cells. Neoplastic cells/ glandular spaces might demonstrate presence of mucin.(12) A cribriform pattern is also considered as a pattern of acinar predominant adenocarcinoma(23)

Papillaryadenocarcinoma is composed of columnar epithelial cells with central fibrovascular cores. This must be differentiated from tangential sectioning of the alveolar walls in an area with lepidic predominant adenocarcinoma. A tumour with a lepidic growth pattern, with the alveolar containing papillary structures, are classified as papillary adenocarcinoma(12)

Micropapillary pattern comprises papillary tufts that lie within alveolar spaces and are enclosed within walls of connective tissue, and lack a fibrovascular core. The tumour cells are mostly small, cuboidal with reversed nuclear polarity and minimal nuclear atypia.(17). Psammoma bodies may be seen. In patients with early stage micropapillary adenocarcinomas the subtype be added as a major histologic subtype owing to its association with poor prognosis in early-stage disease(18)(17)

Solid adenocarcinomas comprises a predominant population of polygonal tumour cells arranged in sheets with no recognizable pattern. If the tumour is 100% solid then intracellular mucin should be demonstrated in atleast 5 tumour cells in each of 2 high power fields and confirmed by histochemical mucin stains.(12)

Colloid adenocarcinoma has dissecting pools of mucin that contain islands of neoplastic epithelium. The epithelium in these cases may be extremely well differentiated and sometimes tumour cells float within the pools of mucin.(12)

Fetal adenocarcinoma comprise of glandular structures with tubules linedby glycogen rich, non-ciliated cells that resemble fetal lung tubules. Sub-nuclear vacuoles are common and characteristic. Sub-nuclear and supranuclear glycogen vacuoles give the tumour an endometrioid appearance.(12)Majority are low grade with a favourable prognosis and high-grade tumours rarely occur. The low-grade fetal adenocarcinomas appear to be driven by mutations in β-catenin, the epithelial cells express aberrant nuclear and cytoplasmic staining by immunohistochemistry(18).

Entericdifferentiation can occur in lung adenocarcinomas, when >50% are classified as pulmonary adenocarcinomas with enteric differentiation. The enteric pattern consists of tall columnar cells exhibiting nuclear pseudo-stratification, prominent nuclear debris and luminal necrosis(12), sharing morphological and immunohistochemical features with colorectal

adenocarcinoma. These tumours should demonstrate atleast one marker with enteric differentiation (CK20, CDX- 2, or MUC2) and are consistently positive with CK7 and TTF-1 in around 50% cases thus help distinguishing from metastatic colorectal adenocarcinoma(18).

The signet ring and clear cell are regarded as cytological features and not as specific subtypes in the new classification since these were associated cytological features seen mainly in association with solid adenocarcinomas and also with other patterns such as acinar, micropapillary or papillary adenocarcinomas(18)

In 1999, the World health organization (WHO) classification introduced the adenocarcinoma of the mixed subtype category, which was retained in the 2004 WHO classification of lung tumours(11)(12)However, a major shortcoming of this classification was that the mixed subtype of adenocarcinoma comprised 94% of cases in one series.(13) Although pathologically accurate, it is of clinical limited utility as most adenocarcinomas fell into this category despite having varied outcomes.(14)(15) Due to the rapidly evolving advances in the field of lung adenocarcinoma, there was an emergent need for improvement in histological categorization of lung adenocarcinomas according to prognosis as compared to the previous classifications.

An international multidisciplinary panel of medical oncologists, pathologists, respiratory physicians, surgeons, molecular biologists and radiologists in 2008, sponsored by the International Association for the study

of Lung Cancer (IASLC), American Thoracic Society (ATS) and European Respiratory Society (ERS), proposed a new classification called the new IASLC/ATS/ERS International Multidisciplinary Lung Adenocarcinoma Classification, published in 2011(19)

IASLC/ATS/ERS Classification of Lung Adenocarcinoma in Resection Specimen.

Preinvasive lesions:

- Atypical adenomatous hyperplasia

- Adenocarcinoma in situ (≤ 3cm formerly BAC).
    - Non mucinous type
    - Mucinous type.
    - Mixed mucinous/non mucinous type
- Minimally invasive adenocarcinoma (≤3cm lepidic predominant tumour with ≤5mm invasion)
    - Non mucinous type
    - Mucinous type.
    - Mixed mucinous/non mucinous type
- Invasive adenocarcinomas
    - Lepidic predominant (formerly nonmucinous BAC pattern, with > 5mm invasion)
    - Acinar adenocarcinoma.
    - Papillary adenocarcinoma.
    - Micropapillary adenocarcinoma.
    - Solid adenocarcinoma.
    - Variants of invasive adenocarcinoma
    - Invasive mucinous adenocarcinoma (formerly mucinous BAC)
    - Colloid
    - Fetal (low and high grade)
    - Enteric.

The new proposed classification proposed the following changes in resection specimens and small biopsies. (18,20)

1) Adenocarcinoma in situ is included as a preinvasive lesion and is suggested for solitarytumours ≤3cm with a pure lepidic pattern and that

lack invasion. These tumours have a 100% disease specific survival on complete resection.

2) Minimally invasive adenocarcinoma (MIA) is proposed for tumours ≤3cm with ≤0.5cm invasion.
3) The term bronchioloalveolar carcinoma is discontinued, and the term 'lepidic' is used for invasive adenocarcinomas, that have a non-invasive component and were previously classified as BAC.
4) Invasive adenocarcinomas are classified on the basis of the predominant subtype after comprehensive histological categorization with 5% increments in each subtype and the mixed subtype has been discontinued. Comprehensive sub typing may be useful in distinguishing multiple primary tumours from intra-pulmonary metastasis which has great impact for staging of tumours.
5) Micropapillary adenocarcinoma is added as a major subtype due to its poor prognosis in early stages of lung cancer.(16,17)
6) The former mucinous BAC is now classified as invasive mucinous adenocarcinoma, excluding tumours that meet defined criteria for mucinous AIS or MIA
7) The clear cell and signet ring adenocarcinoma subtypes are discontinued and are recognized as cytological changes that occur in multiple histological subtypes when present in any amount, however small. There is no recent data available to show clinical significance beyond its association with solid adenocarcinomas.
8) The term mucinous cystadenocarcinoma is found to be rare and are classified as ' colloid adenocarcinomas with cystic change. Hence, the term

mucinous cystadenocarcinoma has been discontinued and included under the category of colloid adenocarcinoma(24)

9) In small biopsies, the classification suggests the use of special stains (mucin and or immunohistochemistry) to classify difficult cases into particular types, namely adenocarcinoma or squamous cell carcinoma(20)
10) Enteric adenocarcinoma although rare, is added as separate subtype to draw attention , as this adenocarcinoma shares few morphological and immunohistochemical features with colo-rectal adenocarcinoma.
11) The fetal adenocarcinomas have been maintained in this classification as the low grade fetal adenocarcinomas are common amongst women, occur in the 4th decade while the high grade fetal adenocarcinomas are most commonly seen in elderly male; suggesting different oncogenic pathways(25)
12) To strategically manage tissue for molecular studies.
13) For judicious use of scanty tissue obtained in patients with lung adenocarcinoma, a few approaches have been mentioned for handling of small specimens.
    i. To cut 10-15 unstained slides, after the presence of tumour is confirmed, so that the block is cut only after routine haematoxylin and eosin staining and adequate tissue is available for immunohistochemistry and molecular workup.

    ii. The other approach is to place the biopsy cores in two separate cassettes for processing such that one block may be used for immunohistochemistry and the other for molecular workup(20).

Targeted chemotherapy based on each cell type and subtype of adenocarcinoma with predictive molecular markers, can thus be made available.

Discontinue Bronchioloalveolar Carcinoma

1. The term BAC used by the 1999 and 2004 WHO classifications have been reclassified into 5 distinct entities in resection specimens and include(18)
2. Adenocarcinoma in situ.
3. Minimally invasive adenocarcinoma.
4. Invasive adenocarcinoma with lepidic component.
5. Invasive mucinous adenocarcinoma, formerly called mucinous BAC.
6. Advanced stage adenocarcinoma with a lepidic component, associated with a very poor survival rate.

Preinvasive lesions:

The 1999 and WHO 2004 classification recognized atypical adenomatous hyperplasia as a preinvasive lesion for lung adenocarcinomas. A major change in the current IASLC/ETS/ARS classification was addition of AISas ‘pre-invasive lesion' for lung adenocarcinomas in addition to previously known atypical adenomatous hyperplasia. Atypical adenomatous hyperplasia and adenocarcinoma in situ are the counterparts to squamous dysplasia and squamous cell carcinoma in situ respectively.

Atypical Adenomatous Hyperplasia :

Atypical adenomatous hyperplasia is a localized proliferation of atypical type II pneumocytes measuring less than 0.5cm, lining the alveolar walls and occasionally respiratory bronchioles(26,27). Gaps are present along the basement membrane, between cells with rounded columnar, cuboidal or peg shaped cells with round or oval nuclei. Atypical adenomatous hyperplasia and adenocarcinoma in situ is a morphological continuum. Differentiation between cellular and cytologically atypical adenomatous hyperplasia and adenocarcinoma in situ may be difficult. Hence multiple characteristics including size, cytological and architectural features need to be evaluated(18).

Adenocarcinoma in situ (AIS):

The diagnostic criteria for adenocarcinoma in situ as described by IASLC/ATS/ERS classification:

1. Small tumours measuring ≤3 cm.
2. Solitary adenocarcinoma.
3. Pure lepidic..
4. No evidenceof stromal invasion, lymphatic, vascular or invasion of pleura.
5. No invasive component (Acinar, solid , papillary, micropapillary, invasive mucinous, colloid, fetal or enteric adenocarcinoma)
6. Absence of intra-alveolar tumour cells.
7. The cell type is non mucinous and rarely mucinous, occasionally resembling goblet cells.
8. Absence of nuclear atypia.

It was suggested that size of the tumour may be underestimated on gross examination, hence correlation is necessary with CT findings is important to determine exact tumour size. Radiologically AIS will present as a ground glass nodule.(18)The presence of solid component should be examined carefully, as these areas may often correlate an invasive component. For a diagnosis of mucinous adenocarcinoma in situ it is important to confirm that the lesion is solitary, well circumscribed and without spread in the adjacent lung parenchyma.

Minimally invasive adenocarcinoma (MIA)

The diagnostic criteria for MIA as suggested by IASLC/ATS/ ERS classification is (18)

1. Small solitary adenocarcinoma measuring**,** $\leq$ 3cm
2. Predominant lepidic growth pattern.
3. Invasive component measuring $\leq$5mm in greatest dimension, in any 1 focus. The invasive component must include any histological subtype other than a lepidic pattern with infiltration into the surrounding,myofibroblastic stroma.
4. The cell type is most commonly non mucinous and rarely mucinous and occasionally resembling goblet cells .
5. In tumours exhibiting multiple microinvasive areas, the size of the largest invasive area is considered, and must be $\leq$5mm .
6. The diagnosis ofMIA is excluded if tumour cells exhibit lymphovascular or pleural invasion or contain foci of tumour necrosis.

Radiologically MIA will have a majority ground glass and a solid component. It is important to note that the diagnosis of AIS and MIA can be made only, if the entire tumour is sampled. Both these tumours should be ≤3cm in greatest dimension.(18)Most of the literature on categories of adenocarcinoma in situ and minimally invasive adenocarcinomas deals with tumours measuring ≤2cm or 3cm and there is insufficient data to support that tumours measuring >3cm would qualify as MIA with 100% disease- free survival. Authors suggest that these tumours should be classified as lepidic predominant adenocarcinoma, with a suspicion of AIS or MIA.(18)

The criteria for AIS and MIA can be applied to multiple tumours if they are synchronous and not intra-pulmonary metastases. A diagnosis of mucinous MIA or AIS should be made with extreme caution as most tumours with this histologic appearance will be invasive mucinous tumours. Tumours diagnosed as AIS and MIA when completely resected have a near 100% disease specific survival(18)

Invasive adenocarcinomas:

In cases of invasive adenocarcinomas, the New International Classification(18) suggests that thorough histologic subtyping should be done for assessments of histological types, semi-quantitatively with 5% increments, thus choosing a single predominant pattern and the percentages of various subtypes should be reported.

There is a hypothesis that lung adenocarcinoma subset can undergo a step wise progression from AAH to AIS to invasive carcinomas, which may be a step-wise process initiated due to multiple genetic changes, responsible for initiation of the malignant phenotype. (22,28)EGFRmutations, KRAS mutations and TTF-1 amplification are characteristic of this progression.(29,29)

Classification for small biopsies and cytology specimens:

70% of lung adenocarcinomas present in advanced stages, and hence are diagnosed on small biopsies/ cytology specimens. Amongst the non-small cell lung carcinomas,most pathologists diagnose well/ moderately differentiated adenocarcinomas or squamous cell carcinomas, but the difficulty persists with poorly differentiated tumours. Hence, these cases 10-30% continue to be diagnosed as NSCLC-NOS(30).

Over the last few years, discovery of EGFRits response to TKI has brought a revolution in the treatment and survival of lung adenocarcinomas. Patients diagnosed as adenocarcinoma or non-small cell carcinoma NOS are eligible for treatment with tyrosine kinase inhibitors . On the contrary, when patients having squamous cell carcinomas who were treated by bevacizumab, developed life threatening haemorrhage. Hence it is important to eliminate a diagnosis of squamous cell carcinoma to determine eligibility of patients for treatment with TKI's (30).

The IASLC/ATS/ERS classification for the first time standardized terminology for lung cancer in biopsies and cytology (20) This group suggested a limited diagnostic workup in order to preserve as much preserve tissue for molecular testing.

Comparison of WHO 2004 classification and the IASLC/ATS/ERS classification for small biopsies and cytology specimens in lung adenocarcinomas(30)

2004 WHO Classification	Small biopsy/Cytology: IASLC/ATS/ERS

Adenocarcinoma Mixed subtype Papillary Solid Acinar	Adenocarcinoma, describe patterns present Micropapillary subtype included. If pure lepidic pattern, add comment: Invasive component cannot be excluded)
Bronchioloalveolar carcinoma. (Non mucinous)	Adenocarcinoma with lepidic pattern (If purely lepidic add note stating that invasive component cannot be excluded)
Bronchioloalveolar carcinoma(Mucinous)	Invasive mucinous adenocarcinoma (describe patterns present).
Mucinous (colloid)	Adenocarcinoma with colloid pattern.
Fetal	Adenocarcinoma with fetal pattern.
Clear cells	Adenocarcinoma with clear cell features (describe patterns)
Signet ring	Adenocarcinoma with signet ring cell features (describe patterns present)
No 2004 WHO counterpart- most solid adenocarcinomas	Morphologic patterns not present- supported by special stainsNSCLC, favour adenocarcinoma.

Squamous cell carcinoma	Morphologic patterns present : Squamous cell carcinoma.
No WHO 2004 counterpart	NSCLC, favour squamous cell carcinoma (by special stains)
Adenosquamous carcinoma.	Morphologic adenocarcinoma and squamous cell differentiation present NSCLC with adenocarcinoma and squamous differentiation. Adeno-squamous carcinoma is a possibility.
No counterpart in WHO 2004	Morphologic patterns of adenosquamous not present, immunostain favour glandular and adenocarcinoma pattern NSCLC, NOS.

Unlike the previous 2004 WHO classification where H&E stain was alone required , the IASLC/ATS/ERS classification advocates the use of special stains (mucin and immunohistochemistry) for exact sub typing of tumours.

The IASLC/ATS/ERS classification (20) proposed that the Non-Small Cell Carcinoma (NSCLC) should be further classified as a more specific histologic subtype, adenocarcinoma or squamous cell carcinoma in small biopsies and cytology specimens.

When clear squamous or adenocarcinoma differentiation is present by morphologic criteria, an established diagnosis of adenocarcinoma or squamous cell carcinoma can be made. It is important to specify if the diagnosis was made on solely on light microscopy or with the aid of special stains (mucin stains /TTF-1/p63). It is also vital to preserve maximum tissue for molecular studies(20)

In NSCLC-NOS lacking morphologic criteria, for adenocarcinoma or squamous cell carcinoma, mucin stains or immunohistochemical markers (TTF-1/p40 or p63) may aid in making the diagnosis. The authors suggest that TTF-1 may be a single value added marker serving both as a pneumocytic marker and marker for primary lung adenocarcinomas, being positive in 75-85% cases. p63 is reported as a valuable marker for squamous histomorphology. CK7 also stains more cases of adenocarcinoma than squamous cell carcinoma.

Cases positive for mucin stains (PASD) and or adenocarcinoma marker (TTF-1) are classified as NSCLC, favour adenocarcinoma.

Tumours positive for squamous markers with moderate and diffuse staining and negative for TTF-1 are classified as NSCLC, favour squamous cell carcinoma.

20%-30% adenocarcinomas are known to display patchy or weak positivity for p63 and have been misinterpreted to favour squamous cell differentiation (31). However, staining patterns for TTF-1 and p63 are mutually exclusive, hence TTF-1 positive tumours are classified as NSCLC, favour adenocarcinoma histology, inspite of the expressionof squamous immunohistochemical markers. Recently polyclonal p40 is demonstrated to be a more specific marker as compared to monoclonal p63, with no overlap with cases of adenocarcinoma(32,33). Thus, studies suggest that a panel of TTF- 1 and p40 may help classify most cases of NSCLC-NOS. Dual nuclear/cytoplasmic markers such as TTF-1/ CK5/6 or p63/Napsin cocktails can also be used to ascertain the diagnosis.

When a dual population of cells, exclusively is positive for TTF-1 or squamous cell markers, a diagnosis of adenosquamous carcinoma can be suggested, although a confirmatory diagnosis can be made only on resection specimen, when each component comprises atleast 10%.

NSCLC-NOS: In case of no clear demarcation on morphology or IHC(20,30).

In few tumours, where no differentiation is established using light microscopy and or immunohistochemistry, the diagnosis of NSCLC-NOS should be made, after cytokeratin staining is performed. If cytokeratin is negative, further stains (i.e., S100, CD45, or CD31) are needed to exclude other tumours such as melanoma, lymphoma, malignant mesothelioma, or epithelioid hemangioendothelioma.

Primary lung adenocarcinomas may also be TTF-1 negative, in this setting, additional immunohistochemistry may be performed (i.e. CDX-2, cytokeratin 20, estrogen receptor, or progesterone receptor) and suggest clinical evaluation to exclude a metastasis from other sites such as the colon or breast.

Invasive mucinous adenocarcinomas or colloid adenocarcinomas are characteristically TTF-1 negative and can be CDX-2 positive, so clinical correlation is needed in such tumours to exclude a metastasis from other sites such as the pancreas or colon.

The authors suggest that a clinco-pathologic correlation is important in cases of NSCLC-NOS. A biopsy obtained from a female with Asian ethnicity and is a non-smoker, with ground glass lesions on imaging, suggests a more likely diagnosis of adenocarcinoma and these patients are likely to be associated with an EGFR mutation.

Distinction of adenocarcinoma from Sarcomatoid carcinomas (22)

A diagnosis of pleomorphic carcinoma, blastoma or carcinosarcoma is not made on small biopsy/cytology specimens. Tumours displaying sarcomatoid features like as nuclear pleomorphism, tumour giant cells, spindled cell morphology are preferably diagnosedas NSCLC, favour adenocarcinoma with giant cell/spindle features. If definite or squamous or glandular differentiation is absent(confirmed by special stains), then a diagnosis of poorly differentiated non-small cell carcinoma with spindle and or giant cells can be made.

Distinction of adenocarcinomas from neuroendocrine carcinomas (30)

A diagnosis of LCNEC is difficult to establish on core biopsies. Cases of NSCLC with neuroendocrine morphology (assessed with neuroendocrine markers: CD56, synaptophysin and chromogranin), a diagnosis suggestive of NSCLC, possibly large cell neuroendocrine carcinoma (LCNEC) can be made. Tumours otherwise diagnosed as adenocarcinoma and lacking neuroendocrine morphology should not be tested for neuroendocrine markers as it would not affect the treatment or prognosis in any way.

The term AIS and MIA should not be used in diagnosis in small biopsies or cytology specimens. The presence of a non-invasive pattern is referred a lepidic.

Histology and prognosis:

Histopathology remains the gold standard for diagnosing lung adenocarcinomas and is an independent predictor of survival in lung adenocarcinomas, independent of the T or the N factor in staging for lung

cancer (34). As per the WHO 2004 classification, 94% of tumours were of the mixed subtype, hence definite typing of tumours was not possible. Since each subtype has a different prognostic value, the IASLC/ ATS/ERS proposed a classification of lung adenocarcinomas, where the tumour was typed on the basis of the predominant subtype, thus helping in prognostication of various subtypes.(18,20).

According to the disease free survival, Yoshizawa (35) identified 3 prognostic groups with low, intermediate and high grade clinical behaviour amongst stage I patients. The low grade comprising of adenocarcinoma in situ and minimally invasive adenocarcinoma with a 100% 5 year disease free survival. The intermediate group included the nonmucinous lepidic predominant, acinar predominant and the papillary predominant adenocarcinomas with a 90%, 84 %, and 83% 5 year disease free survival respectively. The high grade tumours included solid subtype with mucin production, micropapillary, colloid and invasive mucinous and mixed mucinous/ non mucinous subtypes with a 5 year disease free survival of 70%, 67%, 71% and 76% respectively. The poor prognostic group of solid, micropapillary, invasive mucinous adenocarcinoma and colloid predominant subtypes are important because patients with these tumours may be candidates for adjuvant therapy.(36)

Anami et al(37) found that tumour with a lepidic component greater than 50% were associated with a better survival than with tumours with a lesser than 50% component.

A similar study was also done by Sicca et al(38) who also found 3 prognostic groups as discussed above. Several studies have shown that micropapillary carcinoma have a poor prognosis.(16,35) Miyoshi et al studied 344 patients, and histologically divided them into 2 group namely micropapillary (n= 139, 40%) and others (n = 205; 60%). They found the micropapillary subtype was significantly more associated with lymph node metastasis, intrapulmonary metastases, pleural invasion and non-smoking status, with a significantly lower 5 year survival of 79% in contrast to 93% of the non-micropapillary group(17).Ohtaki et al found that patients with solid adenocarcinoma (SAC) had significantly poorer prognosis than patients without SAC, irrespective of the SAC ratio(39). Noriko et al found that solid predominant adenocarcinomas showed a strong association with higher grade, poorly differentiated tumours having a significantly poor survival as compared with tumours with moderate and well differentiation ($P<0.001$) and were associated with a poor prognosis as compared to the non-solid subtypes ($P=0.001$). These tumours also correlated with a larger tumour size and were common amongst smokers and were not commonly associated with EGFR mutations (5)

Okada and Hashimoto et al(40)proposed a prognostic cell type classification, which included the hobnail, columnar/cuboidal, polygonal, goblet and mixed cell types and found that it was the hobnail type which was significantly associated with EGFR mutations ($P<0.001$), followed by mixed, columnar/ cuboidal, polygonal and goblet cells. The percentage of smokers was significantly higher amongst the cuboidal/ columnar and polygonal cells

in contrast to the hobnail and the mixed cell types. The 5 year survival rate for stage I, by the 5 cell classification was highest in the hobnail cell type (83%), followed by polygonal (80%) > columnar/ cuboidal (74%) and goblet cell types; amongst stage II-IV, the polygonal cell type (64%) had a better prognosis, followed by hobnail (41%), mixed (39%) and cuboidal/columnar (24%). This contrasts other studies wherein the polygonal cell type had a worse survival in comparison to the other cell types.

Okada and Hashimoto also compared the five cell type to TTF-1 and found that TTF-1 positivity was ≥ 50% and ≤50% in 74% and 26% cases respectively, similar to a study by Yatabe et al who found a positivity of ≥ 50% and ≤50% in 72% and 28% respectively. Okada and Hashimoto found that the hobnail cell type was consistently associated with TTF-1 positivity in almost all cases (99%) , mixed cell type (89%) and in contrast the cuboidal/columnar cell type (54%) and the polygonal cell type (50%). Considering this outcome, they developed a hypothesis that the carcinoma cells imitate characteristics of progenitor cells i.e. almost all hobnail cells develop at the terminal respiratory unit (TRU), the mixed more distal to than that of the terminal respiratory unit and the remainder (cuboidal/columnar/polygonal) develop at the junction of TTF-1 positive and negative bronchioles, bronchi and bronchial glands.

Presence of individual histological features like invasion, desmoplasia, stromal elastosis, and lymphovascular invasion were prognostic indicators. Invasion has been defined as presence of acinar, papillary, micropapillary patterns or myofibroblastic stroma associated with invasive tumour or if the

tumour displays lympho-vascular invasion, invades pleura or contains tumour necrosis.(19)

Xu et al in his study identifiedseveral types of invasion, i) invasion with areas of destruction of the alveolar pattern with relatively uniform acinar structures and without a desmoplastic response. ii) Invasion of orderly acini with associated desmoplasia comprising of reactive fibroblasts. iii) invasion with a desmoplastic response, compressed acinar structures or single tumour cells iv) Elastosis may occur in areas of lepidic spread, being prominent in central scar without invasive glands .(41) However, Noguchi et al in his study separated alveolar collapse with elastosis from invasion with fibroblast proliferation, emphasizing the good prognosis associated with the former.(42) The Noguchi classification reported that the absence of invasion imparted a benign prognosis to the tumour. Based on the latest IASLC/ATS/ERS classification, it is the size of invasive component which is a cut off between low grade and intermediate grade tumours. (18) Tumours with less than 5 mm invasive component are considered well differentiated neoplasm’s and behave like non-invasive pure BAC's, recently described as AIS and MIA.

Several studies suggest that the invasive tumour size is an independent prognostic factor, and it may be a better predictor of prognosis than overall tumour size in lepidic predominant tumours(43)(44). Recently Xu *et al*(45) described invasive components of lepidic predominant tumours by 3 patterns, i) complex acinar papillary, defined by enlarged acini with no identifiable alveolar architecture, the septae between the complex patterns

being about the same thickness like alveolar space between lepidic growth (40-60mm), ii) invasion with elastosis and desmoplasia, acinar pattern with open lumina and ii) invasion with desmoplasia and presence of compressed glands or single cell invasion. Xu et al(45) also stated that it is not the amount of invasion but the type of invasion which determines prognosis. In their study of lepidic predominant adenocarcinomas, they found that tumours in the absence of lymphovascular invasion, solid or single cell invasion had a good prognosis inspite of a large invasive area, including an elastotic scar. Invasion with desmoplasia with compressed and single cell growth is associated with worse prognosis compared with other patterns of invasion(45).

Noriko Motoi found that there was a strong correlation of survival with stage and grade. 5 year survival was 81%, 62%, 0% respectively in stage I, II and III respectively (P<0.001). The 5 year survival of these patients with poorly differentiation tumours showed 37% 5year survival compared with 84% in well or moderately differentiated tumours. (<0.001). (5) A study by Yoshizawa et al showed that higher stage,male gender, necrosis, poor differentiation and vascular invasion were all associated with worse disease-free survival. (46)

Epidemiologic research has found evidence that chronic inflammation can initiate or promote development of lung cancer in conjunction with tobacco use .(47–49)

A number of studies have validated the IASLC/ARS/ETS classification, thus emphasizing sub typing of lung adenocarcinomas, in view of prognostic implications. (41,50,51)

Grading of adenocarcinomas:

In the current setting there is no well-established histologic / cytologic grading system for the diagnosis of lung adenocarcinomas. The overall grade is determined by the worst grade of the tumour. Recently, architectural patterns have suggested prognostic outcomes; poor (solid/micropapillary), intermediate (papillary and acinar), favourable prognosis (non mucinous lepidic predominant). Hence comprehensive histologic sub typing of invasive tumours may will be a simple way to grade these tumours.

Immunohistochemistry:

Differentiating primary adenocarcinomas from metastatic adenocarcinomas in the lung can be challenging. TTF-1 is a valuable and favoured marker in the diagnosis of lung adenocarcinoma, with specificity and sensitivity being positive in ~80% of primary lung adenocarcinomas(52). Most pulmonary adenocarcinomas have a TTF-1 positive, CK7 positive and CK20 negative immunophenotype. One exception is invasive mucinous adenocarcinomas that are TTF-1 negative, positive for CK7, frequently CK20and CDX-2 can be positive. Hence clinical correlation may be needed to exclude metastasis from pancreas, colon. (12) Metastatic adenocarcinomas with the exception of carcinomas of thyroid origin are negative for TTF-1.

Negative mucin stains and positive staining for thyroglobulin helpseparate metastatic thyroid carcinoma from an adenocarcinoma of the lung.

Napsin is a recently developed marker for the diagnosis of primary and metastatic adenocarcinomas.Napsin A (Nap-A)a functional aspartic proteinase is a more specific and sensitive alternative for primary and metastatic lung adenocarcinomas. A study by Kim et al found that the positivity and expression of Nap-A in metastatic lung adenocarcinomas was better as compared to TTF-1. In contrast there was no significant difference between Nap-A and TTF-1 in primary tumours of the lung. (53)Most non pulmonary adenocarcinomas are negative for Nap-A except, clear cell adenocarcinomas of the ovary, uterus and renal cell carcinomas. Thus, the combined use of Nap-A and TTF-1 results in increased specificity and sensitivity for diagnosis of primary lung adenocarcinomas(32).

Polyclonal p40 is a specific marker as compared with the monoclonalp63 forsquamous cell malignancies without overlap inadenocarcinomas, suggesting that p63 may be replaced by monoclonal p40.(20).

Epidermal growth factor receptor and mutations:

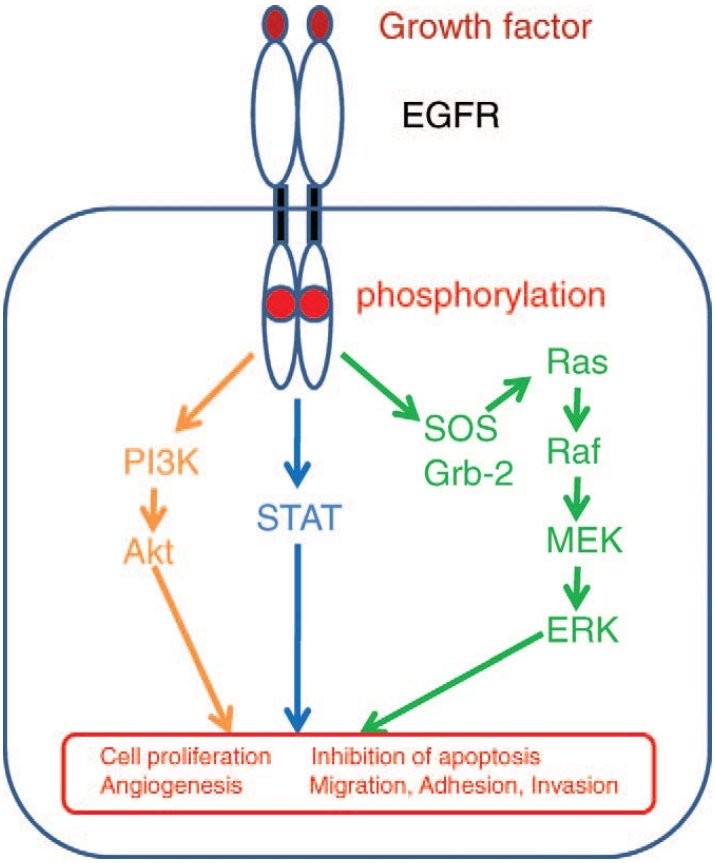
Lung adenocarcinomas are heterogeneous, with different pathogenesis in subtypes, reason being one subtype common in smokers and associated with KRAS mutations, the other otherbeing common in non-smokers and is associated with EGFR mutations. The EGFR and KRAS mutations are always mutually exclusive in occurrence.(3).

Lynch et al reported that a subgroup of cases with non-small cell carcinoma , commonly with adenocarcinoma, harboured a specific activating mutation in the EGFR gene that correlated with clinical response to the tyrosine kinase inhibitor namely Gefitinib.(54). This was subsequently confirmed by other investigators and showed consistently that mutations in the tyrosine kinase domain of epidermal growth factor receptor were more common amongst Asians, women, never-smokers and adenocarcinomas. (54)(55)(6). These data suggest that the pathogenesis of non-small cell carcinoma in never-smokers could be different from the smokers. The development of targeted therapies, specially tyrosine kinase inhibitors, that act as competitive inhibitors of the ATP binding pocket and thereby block the downstream signalling pathway, have provided improvements in the therapeutic response, thus highlighting the clinical benefits of identifying and targeting biologically relevant alterations(6).

EGFR is a transmembrane receptor tyrosine kinase protein expressed in normal epithelial, mesenchymal and neurogenic tissues.(56). It is associated with tumorigenesis in several malignancies including NSCLC. (57)(58)EGFR belongs to the family of 4 homologous receptors, EGFR and

(ERBB2-4), (59). Although their basic structures are quite identical, each one has distinct properties, including variations in tyrosine kinase activity.

EGFR has an extracellular ligand binding domain, a transmembrane portion, intracellular tyrosine kinase and regulatory domains. Upon binding of a specific ligand (e.g. epidermal growth factor), the normally functioning EGFR undergoes conformational change and phosphorylation of the intracellular domain occurs, leading to downstream signal transduction by various pathways. The tyrosine kinase receptor transmits signals of from the extracellular to intracellular region, thus transmitting stimuli by signal transduction to the nuclei. The PI3K/akt pathway,RAf/Ras/MAPK pathway and the JAK/STAT pathways are important signalling pathways for EGFR. Signal transduction causes enhancementof cell differentiation and proliferation. The Ras/Raf/ MAPK pathway enhances cellproliferation and cell survival. The P13K/Akt pathway is associated with cell growth, invasion and inhibits apoptosis. Hence, depending on the pathway involved , the end result is cell proliferation or cell maintenance by inhibition of apoptosis(60).



EGFR and downstream signalling pathways

The EGFR gene is over expressed in various tumours including tumours from the head and neck region, lung, renal breast, colon, ovary, prostate, gliomas, pancreas and bladder tumours with good prognostic clinical significance for cervical, ovarian and head and neck cancers and low prognostic value for non-small cell lung carcinomas(60).

Somatic mutations in the TK domain of EGFR are a common feature observed in lung adenocarcinomas,(61) and are exploited as a therapeutic and prognostic tool in treatment of lung cancers with tyrosine kinase inhibitors

such as gefitinib and erlotinib, (62)(54). These drugs act by blocking the cell signalling as they bind to the tyrosine kinase domain. The presence of these mutations leads to activation of the signal transduction pathways, leading to anti-apoptosis and cell proliferation, regardless of the presence of extracellular ligand. (63) In addition to mutations in EGFR, there is evidence that increased EGFR gene copy number, defined as high polysomy or amplification is also associated with a better response to TKI's. Some cases of adenocarcinoma may show both EGFR mutations and increased gene copy number while others may show only one of them. Studies have shown that approximately 50% of EGFR mutated cases may show an increased EGFR copy number, while about 75% with increased copy number have mutations. (56) DNA mutations in EGFR as detected by polymerase chain reaction (PCR) can occur in regions corresponding to the extracellular or intracellular portions of the protein.

Although several mutations have been found, the most activating mutations are found within exons 18, 19, 20 and 21 of the EGFR TK domain(64). Small in‑frame deletions in exon 19 around codons 746-750 and a point mutation (CTG to CGG) in exon 21 at base pair 2573, resulting in the substitution of lucine by arginine in codon 858 (L858R) makes up for approximately 90 % of all these mutations. (3,65). Point mutations in exon 18 and 20 are described in about 5% of patients with non-small cell lung carcinoma. Several other mutations have are also found at lower frequencies ; however, their significance is not yet deduced (66).These mutations cause change in the ATP binding domain, resulting in continuous activation of

EGFR, without ligand binding. This affinity is down regulated by Gefitinib, a tyrosine kinase inhibitor so that cancer cells are susceptible to apoptosis, thus causing reduction in cancer size.

The in-frame deletions of exon 19 are significantly more frequent in males, and point mutations in exon 21 occur more frequently in females(3) A study by Jackman et al has shown that EGFR exon19 deletions have a longer survival, with Gefitinib or Erlotinib compared with those having the L858R mutation (67)(68).

The prevalence of EGFR mutation data has found to vary in ethinic populations ranging from 24% to 66.3% amongst East Asians(69)(70)(71) to about 10% in the European and the North American populations.(72) The prevalence of EGFR in India ranges from 25.9 -51.8% .(73)(74)(75)EGFR mutations are found to be significantly more common in tumours expressing thyroid transcription factor-1 (TTF-1), hence TTF-1 positivity can predict higher EGFR mutation in lung adenocarcinomas(76)(77)(5).

A correlation study between IASLC/ATS/ERS lung adenocarcinoma classification and molecular changes revealed that EGFRmutations are associated with a high frequency of AIS, MIA , lepidic predominant and papillary predominant subtypes (85.7% AIS, 83.3% MIA, 71.4% lepidic, and 68.5% papillary), followed by acinar (38.4%) and micropapillary (40.1%) predominant subtypes; whereas they were uncommon in the solid predominant

and mucinous BAC subtype(78)(79). (80–82) Motoi N et al found that the presence of papillary and micropapillary adenocarcinomas strongly correlated with EGFR mutations (P<0.001). (5) The study also found that majority cases of major solid subtype, lacked EGFR mutations (P=0.01) A few studies have reported intratumoural heterogeneity of EGFR mutations(83)(84) A comprehensive study by Yatabe et al (85) suggested that intratumoral heterogeneity of EGFRmutations is very rare, and pseudo heterogeneity is s due to a combined cause of mutant allele-specific imbalance and heterogeneously distributed EGFRamplification.

The current recommendation for candidates eligible for EGFR mutation testing and treatment with TKI's are patients diagnosed as:

1. Adenocarcinoma
2. Non-small cell carcinoma, favour adenocarcinoma.
3. Non-small cell carcinoma- NOS.

Studies have shown that adenocarcinomas with EGFR mutational analysis have a better overall survival as compared to those without them. (86,87). Hence, when the diagnosis is equivocal, pathologists must be aware that a diagnosis of squamous cell carcinoma / NSCLC, favour squamous cell carcinoma will exclude these patients from molecular testing and targeted chemotherapy. In these circumstances, it is best to favour a diagnosis of NSCLC-NOS, thus making the patient eligible for targeted therapy.

Primary tumours or metastatic lesions are equally suitable for EGFR testing. Amongst patients with multiple or apparently separate primary lung adenocarcinomas, each tumour may be individually tested and testing of multiple different areas within a single tumour is not necessary (88).

The presence of an EGFR sensitizing mutation is the biomarker most strongly associated with progression-free survival benefit from first-line EGFR TKI treatment over chemotherapy. However, these patients with EGFR-mutant lung adenocarcinoma develop progression of disease on TKI therapy after a median of 10 to 16 months and condition has been described as "acquired resistance"(89). In approximately half of the cases, tumour cells obtained after disease progression contain a second site mutation in the EGFR kinase domain The most common (>90%) lesion involves a C →T change at nucleotide 2369 in exon 20, which substitutes methionine for threonine at position 790 (T790M).(90).

A number of theories have emerged as to how tumour cells with the T790M mutation emerge within TKI treatment. Sub clones bearing may arise de novo, while on treatment. Crystal structure modelling has demonstrated that residue T790 is located in the ATP-binding pocket of the catalytic region of EGFR, and is critical for the binding of TKI'S.(91) However, based on similar studies in Chronic myelogenous leukaemia, it is a possibility that in cases of NSCLC sub clones bearing this secondary mutation pre-exist within the primary tumour clone in individual patients, however at low frequencies (61).

Gefitinib-sensitive lung cancers are known to develop resistance dueto focal amplification of the MET proto-oncogene. Amplification of MET causes resistance by driving the ERBB3 (HER3)–dependent activation of PI3K pathway which is specific to the EGFR/ERBB family receptor. Hence, MET amplification is an example of the resistance mechanism characterized by gene amplification of a kinase that is not a direct or downstream target of Gefitinib or Erlotinib (92).

Irreversible EGFR inhibitors, which are currently under clinical development as treatment for patients whose tumours have developed acquired resistance to gefitinib and erlotinib, may be ineffective in the subset of tumours with a MET amplification even if they contain an EGFR T790M mutation. Therefore, combination therapies with MET kinase inhibitors, which are in early-stage clinical trials, and irreversible EGFR inhibitors should be considered for patients whose tumours have become resistant to gefitinib or erlotinib.(92) Notably, a small percentage of NSCLCs from EGFR TKI–naïve patients have been reported to contain both an EGFR-activating mutation and MET amplification(93)(94). This situation is analogous to the observation that untreated NSCLCs occasionally have an EGFR T790M. These concurrent genetic alterations may help explain why some NSCLCs with EGFR-activating mutations fail to respond when initially treated with gefitinib (95).

Phase 3 trials on patients with lung adenocarcinomas / adenocarcinoma component found that patients with EGFR mutations have better treatment

outcomes when treated with TKI's than with conventional platinum based chemotherapy. A study at our centre, by Bhat et al found that patients with a positive EGFR mutational status showed a better progression free survival in those who received both chemotherapy followed by TKI as compared with the EGFR positive subset who received only TKI's. Thus, this study supports the concept of maintenance therapy in an Indian cohort (73).

Thus multiple phase 2 clinical trials have found that EGFR mutations are predictive of the beneficial response to TKI and have a progression free survival(30).

Histologic subtypes of lung adenocarcinoma with molecular and radiological correlation(22)

SUBTYPE	MOLECULAR FEATURES	CT SCAN
Non mucinous AIS and MIA	TTF-1 + (100%) EGFR mutations and never smokers (10-30%) KRAS mutations and smoker (10-30%)	GGN, solid nodule
Lepidic (non mucinous)	TTF-1 + (100%) EGFR non-smokers (10-30%) KRAS mutation, smokers (10%) BRAF-5%	GGN with solid nodule or solid nodule
Acinar	TTF-1 positive or negative. KRAS mutation smokers (20%) EGFR mutations <10% non-smokers. EML4/ALK translocation >5%	Solid nodule
Solid	TTF-1 positive (70%) KRAS mutationand smokers (10-30%) EGFR mutations in non-smokers (10-30%) EGFR with amplification 20-50% MUC1 positive EML4/ALK fusion gene>5% P53: 50%.	Solid

Papillary	TTF-1 positive (90-100%) EGFR mutation: 10-30% KRAS- lack KRAS (3%) ERBB2-3% P53- 30%.	Solid nodule
Micropapillary	KRAS mutations (33%) EGFR mutations (20%) BRAF mutations (20%)	Not known
Invasive mucinous adenocarcinoma	TTF-1 rare positive (0-33%) KRAS 80-100% Negative for EGFR mutations. Muc2+ Muc5+ Muc 6+	Consolidation

KRAS (Kirsten Rat Sarcoma Viral Oncogene Homolog) mutations:

KRAS mutations are reported in 8-30% by various clinical trials in cases of non-small cell lung cancers(3) Activation of KRAS mutations leads to initiation of MAPK pathway thus regulating cell proliferation and apoptosis. The PI3K pathway regulates cell survival and anti-apoptotic responses. Majority of KRAS mutations are found in codons 12 and 13. KRAS mutations are commonly seen amongst smokers/ asbestos exposure, non-Asians and invasive mucinous adenocarcinomas. The solid predominant subtype has also found to be associated with KRAS mutations Patients with KRAS mutations may not derive benefit from TKI'S or platinum based chemotherapy. The presence of EGFR and KRAS mutations is mutually

exclusive. EGFR or KRAS negative tumours may have EML4-ALK mutation(3)

Lung cancers with ALK translocation (Human Echinoderm Microtubule- Associated Protein-Like-4- Anaplastic ymphoma Kinase).

The EML4/ALK translocation has been recently identified as a predictive biomarker in patients with NSCLC, leading to oncogenic constitutive activation of ALK. A minority of these tumours harbour a inversion in chromosome 2p, giving rise to the EML4-ALKfusion gene. There are no activating mutations in this fusion gene, dimerization of the fusion protein is the cause for its activation. The ALK mutations have an intrinsic tyrosine kinase activity with downstream signalling independent of EGFR mutations. ALK translocations occur in approximately 5% of lung carcinomas, adenocarcinomas. Young men, light ornon-smokers and an advanced stage of disease may identify a population at risk to harbour this translocation. ALK rearrangements are seen with solid histology with signet ring cells among the western population (3)Tumours with EML4-ALK mutation are TTF-1 positive and may also be positive for p63. Tumours with EML4-ALK mutations are found to be mutually exclusive with KRAS and EGFR mutations. Denovo resistance is also known to develop in the domain of the EML4-ALK fusion gene, reported during the treatment with ALK inhibitor .(96)

Vascular Endothelial Growth Factor:

Angiogenesis is an important patho-physiologic event required for tumour growth and survival. The VGEF signalling pathway important in vascular permeability, endothelial cell migration, proliferation and cell survival. VEGF is strongly expressed in NSCLC and correlates with increased microvascular density and poor clinical outcome(3).

ROS-1 rearrangements:

Recently ROS-1 rearrangement has been found in approximately 1.7% if lung adenocarcinomas, with effective targeted molecular therapy. These are mutually exclusive with ALK rearrangements, seen amongst the young, never smokers and adenocarcinomas. However, there has been no particular histologic subtype found in association with it (3,20)

BRAF mutations:

BRAF gene mutations are also seen in lung adenocarcinoma. BRAF a member of the Ras/MAP kinase pathway is downstream of KRAS phopshorylating MEK and ERK, culminating in genes favouring proliferation and survival. V600E (valine to glutamate substitution) is the most common BRAF mutation in lung adenocarcinomas, seen in heavy smokers. 1-3% of BRAF mutations have been identified in lung adenocarcinomas, however these mutations are more common in colorectal cancers. BRAF mutations are also exclusive of EGFR, KRAS and ALK mutations.(3)

Other rare mutations: The PIK3CA/AKT, c- Met, KIF5B-RET fusions and Her2neu are rare mutations associated with non-small cell carcinomas(3)

Pyrosequencing:

The direct DNA sequencing is the most common and conventional method for detection and identification of mutations in tumours. However, for the test to be reliable the mutant DNA must comprise atleast 20% DNA. Pyrosequencing is a non-electrophoretic real time sequencing technology with luminometric detection.

Pyrosequencing has the following advantages over Sanger sequencing:

1. The pyrosequencing technique has higher sensitivity.
2. The Sanger sequencing technique needs greater than 20% tumour volume for a reliable result, whereas a tumour load of 5% would suffice for pyrosequencing. Hence, pyrosequencing has higher sensitivity.
3. Pyrosequencing is much faster and cost effective than the Sanger Sequencing.
4. However, the disadvantages for pyrosequencing are that data analysis may be complex at times and it can sequence only a short length of nucleotide sequence(97).

Studies have validated the new International classification and confirmed the prognostic value amongst histological subtypes of lung adenocarcinoma, including the low grade AIS, MIA, lepidic predominant and

the intermediate grade acinar and papillary adenocarcinomas, high grade included invasive mucinous, solid, colloid, micropapillary adenocarcinomas(51,98).

In this era of personalized treatment a relationship has been established between histological types of lung adenocarcinoma and EGFR mutational status. Thus, histopathological sub typing of tumours according to the new classification is a potential way to stratify patients according to prognostic significance and specify treatments for targeted therapy.

Materials and Methods

The study was performed in the Department of General Pathology, Christian Medical College, Vellore, India, approved by the Institutional Review Board. We evaluated a retrospective series of all Primary Lung Adenocarcinomas from June 2011- December 2012 using the archival stained and mounted Haematoxylin and Eosin (H&E) slides and formalin fixed paraffin embedded blocks (FFPE). The cases were retrieved using the Oracle based Pathology workstation. Cases were categorized on the basis of the current classification by the International Association Society of Lung Cancer, American Thoracic Society and European Respiratory Society (IASLC/ATS/ERS)(18,20). Retrospective data regarding the relevant history, clinical diagnosisand radiological findings were obtained from the clinical workstation of the institution. EGFR mutational analysis was performed in Molecular Pathology Department.

380 cases with a diagnosis of Primary Lung Adenocarcinomas were retrieved from June 2011- December 2012.

274 cases were included.

106 cases were excluded due to one or more of the following reasons:

Inclusion criteria:

1. All cases of Primary Lung Adenocarcinoma biopsied in our institution from June 2011- December 2013.

Exclusion criteria:

1. Slides and blocks for review, referred for opinion by other hospitals or institutions.
2. Inadequate tissue for definite categorization of tumour.
3. Morphological features suggestive of large cell carcinoma and negative for TTF-1.
4. Morphological features suggestive of Poorly Differentiated Carcinoma and negative for mucin stains and TTF and or CK7.
5. Squamous cell carcinoma.

Clinical parameters: Demographic details including age, gender, smoking status and geographical location were obtained from the electronic computerized medical records.

Radiological parameters: Radiological findings including tumour laterality, location, maximum tumour dimension, associated lymphadenopathy and stage were obtained from the CT scan results were obtained from the Picture Archiving and Communication System (PACS).

Histological parameters: A detailed histopathologicalstudy was done by two pathologists, who were blinded to clinical outcomes. Individual features including the predominant cell type, presence of mucin, desmoplastic response, stromal elastosis, lymphovascular invasion, necrosis, inflammatory response and tumour grade were studied. The WHO 2004 grading system was used. Comprehensive histologic sub typing was done to assess histologic

patterns semi quantitatively in 5% increments, thus choosing a single predominant pattern using the current IASLC/ETS/ARS classification. These features were correlated with one or more immunohistochemical markers namely TTF-1, CK7, CK20 and BerEp4 were performed using the automated Ventanna system .

EGFR mutational status was performed on a subset by PCR Sequencing and the results were correlated with final histologic diagnosis, clinico-pathologic parameters, including age, gender, location, smoking status and stage of disease. All the patients were staged based on the American Joint Committee on Cancer (AJCC) TNM staging manual, 7th edition. This study did not include treatment strategies used and also did not include follow up of cases.

Immunohistochemistry for TTF-1/CK7/CK20/BerEp4

Immunohistochemistry was then performed on Formalin fixed and paraffin embedded tissues (FFPE) tissue using the monoclonal antibody in 263 cases for TTF-1, CK7 and CK20 and BerEp4 when requested for.

Protocol for automated immunostaining:

Paraffin embedded tissue sections were cut at 4μ thickness and floated in poly L-lysine coated slides and incubated overnight at 37˚C.

These slides were then treated with 4% milk solution for 10 minutes to eliminate the hydrophobic effect and give positive charge to the slides

Then the slide labels were bar coded and the labeled slides were loaded in Ventanna Benchmark XT autostainer (a fully automated immunostainer).

Individual protocols have been designed in the software attached to the machine for each marker. Specific protocols were selected according to the marker (table below)

A standard protocol was used for most of the markers with a minimal variation for few individual markers. The steps included in this protocol were as follows:

Deparaffinization

Liquid coverslip application.

Heat induced antigen retrieval by treating with standard CC1 solution (pH patent with the company) for one hour at 90˚C.

Then the primary antibody was added and incubated for 40 minutes @ 37˚C.

Then the secondary antibody (Multimer) was added and incubated for 8 minutes.

Finally the slides were counterstained with Haematoxylin and incubated for 8 minutes, followed by incubation with the bluing reagent for 4 minutes.

(From antigen retrieval till counterstaining, in between every step the slides were washed with reaction buffer. The whole process is automated).

Then the slides were brought to 80% alcohol (2 changes) to remove the liquid coverslip and then dried and mounted in DPX.

A known positive control slide was added to each batch. A strong nuclear staining of tumour cells was considered positive.

Antibody	Protocol	Dilution	Clone	Manufacturer
TTF-1	STD 40	1:200	8G7G3-1	Dako
CK7	STD 40	1:50	OV-TL12/30	Dako
CK20	STD 32	1:100	KS20.8	Dako
BerEP4	MILD 32	1:100	BerEP4	Dako

DNA extraction, PCR amplification and DNA sequencing:

Formalin fixed and paraffin embedded blocks of patients with Primary lung adenocarcinomas were retrieved from the archives of Pathology. The H&E sections were examined by the investigator and the Guide and the area with maximum tumour cellularity was identified, preferentially >50%. The tumour was manually micro dissected if non-neoplastic lung parenchyma was identified. About 3-4, 10 µ sections were taken for DNA extraction. Extraction was carried out using the DNA tissue extraction kit from QIAGEN India Pvt. Ltd, New Delhi (described below).

DNA extraction:

In a 2 ml tube, the scrapped tumour from glass slides is scrapped and added to 200 µl ATL buffer.

20 µl Proteinase K was added to the microcentrifuge tube mixed by vortexing and incubated in a shaking waterbath at 56^0C overnight until the tissue was completely lysed.

200µl of buffer AL was added to the sample and mixed by pulse vortexing for 15 seconds, followed by incubation at 70^0C for 10 minutes.

Tubes were briefly centrifuged to remove drops from the lid.200 µl of 100% ethanol was added to the sample and mixed by pulse vortexing for 15seconds. After mixing, the 1.5ml microcentrifuge tube was centrifuged briefly to remove drops from the lid.

The mixture from step 4 (including the precipitate) was carefully transferred to the QIAamp spin column in a 2ml collecting tube. Cap was closed and centrifuged at 6000g (8000 rpm) for 1 minute. QIAamp spin column was then placed in a clean 2ml collecting tube and the filtrate was discarded.

500 µl of buffer AW1 was added to QIAamp spin column without wetting the rim. Cap was closed and centrifuged at 6000g (8000 rpm) for 1

minute. QIAamp spin column was placed in a clean 2ml collection tube and the collecting tube containing the filtrate was discarded.

500 µl of buffer AW2 was added to QIAamp spin column without wetting the rim, followed by centrifugation at 20000g (14000 rpm) for 3 minutes.

QIAamp spin column was placed in a clean 2ml collection tube and the collecting tube containing the filtrate was discarded. The spin column was centrifuged at full speed for 1 minute.

The spin column was placed in a clean 1.5ml microcentrifuge tube and the collecting tube containing the collection tube was discarded. 200µl of buffer AE was added to the spin column, followed by incubation at room temperature for 5 minutes. The spin column was centrifuged at 6000g (8000 rpm) for 1 minute.

1.0µl of the DNA sample was used for quantification using the Nanodrop (NanoDrop technologies) and the 260/280 ratio was determined. Measurements were repeated twice for confirmation.

If PCR was not carried out immediately, samples were stored at -70^{0}C and taken only just before the PCR procedure.

PCR amplification: The polymerase chain reaction (PCR) for four exons, namely 18, 19, 20 and 21 was performed using the published primer sequences used by Lynch et al (54). All reactions were carried in 25µl volume. DNA sample and the reagents were thawed to room temperature before starting the reaction. Tubes were tapped and centrifuged to remove drops from the sides and lid.

Master Mix was prepared in 0.6ml PCR tubes by mixing 2.5µl buffer, 2 µl DNTPs (Fermentas, USA), 2µl of 20 picomoles of forward and reverse primers (Sigma Aldric India), 0.3µl of Taq polymerase (Takara, Japan) and 14.2 µl of distilled water. The tubes were tapped and centrifuged to mix the reagents thoroughly.

23 µl of the master mix was added to the respective 0.2ml PCR tubes. The PCR tubes were then transferred to the amplification area and 2µl of optimally diluted DNA containing 50-80ng of DNA was added to the respective PCR tubes. A non-template control (NTC) was also run with every batch of PCR reaction for all four exons 18, 19, 20 and 21. 2µl of distilled water was added to the NTC tubes.

The PCR tubes were tapped and centrifuged before loading into Veriti thermal cycler (Applied Bio systems, USA). The following thermal cycling profile was followed for all PCRs: 95^0C for 8 min, 95^0C for 1 min, optimized anneal for 1 min, 72^0C for 1 min and final extension of 72^0C for 10 min. PCR

products were detected by gel electrophoresis on 2% agarose gel (SRL, Cisco Research laboratories, India).

Once the PCR products were amplified, the product was cleaned to remove unwanted PCR fragments and unused reagents. The products were cleaned based on the principle of ultra-filtration. 15 µl of PCR product and 85ul of sterile water were applied onto the ultra-filtration membrane of the wells of the pre-clean plates (Millipore/Merck, USA). 20 Hg pressure was applied for 10 minutes. 100µl of sterile water was then added into the well and 20 Hg of vacuum was applied for 10 minutes. The PCR products were eluted out with 30µl of sterile water.

The PCR product was detected using a 2 % agarose gel. Sequencing of both the sense and antisense strands for all 4 exons was performed with an automated DNA sequencer (ABI PRISM 310 genetic analyser) using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Bio systems, USA). Mutational analysis was performed by comparing the sequence with the wild type and by looking for the presence all known mutations in these exons.

DNA Sequencing: Once good pre clean products were obtained on electrophoresis, sequencing of both the sense and antisense strands for all 4 exons was set up with an automated DNA sequencer (ABI PRISM 310 genetic analyser) using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Bio systems).

Sequencing PCR was carried at 10 µl volume. Master mix was prepared using 1 µl of buffer, 0.3 µl of RR mix, 5.1 µl of distilled water, 1.6 µl of 1 picomole of forward or reverse primers and 2 µl of optimally diluted pre clean product. The following thermal cycle was used for sequencing PCR: 25 denaturationcycles for 15 sec at 96^0C , with annealing at 50^0C for 20 sec and followed by extension at 60^0C for 4 min.

Post clean-up was done using membrane based ultrafiltration using injection solution (Millipore/Merck, USA). 10 µl of sequence product was mixed with 30 µl of injection solution. 20 Hg of pressure was applied for 10 min. Then 40 µl of injection solution was added and a vacuum of 20 Hg was applied for 10 minutes. Sample was eluted out with 30 µl of injection solution.

Samples were loaded onto 96 well plates and sequencing was done with an automated DNA ABI 3130 Genetic analyser. Mutational analysis were performed and reported by comparing with the wild type and by looking for the presence of mutations at the respective codon.

Statistical analysis:

Descriptive statistics for continuous data was analyzed using mean withStandard deviation or median with inter-quartile range. Categorical data was described usingFrequencies and percentages.

Histopathological subtypes with EGFR mutational analysis wasassociated using Fischer's exact test and Chi square test.

P values < 0.05 was considered statistically significant in this study. Data was analyzed using the Statistical Package for Social Sciences (SPSS) software (Windows version 16).

RESULTS:

The study included 274 patients with Primary lung adenocarcinomas. Majority patients were males 67.88 % (n=186) and 32.12% (n=88) were females with the mean age being 58 and 55 years respectively. Although our cohorts were mainly from the East 148/274 (54%) and Southern parts of India 93/274(33.90%), the demography did not yield any significant results (P =0.254). A minority of patients were from the North 6(2.2%), West 2(2.2%) and 25(9.1%) from foreign countries mainly Bangladesh **(Figure 1).**

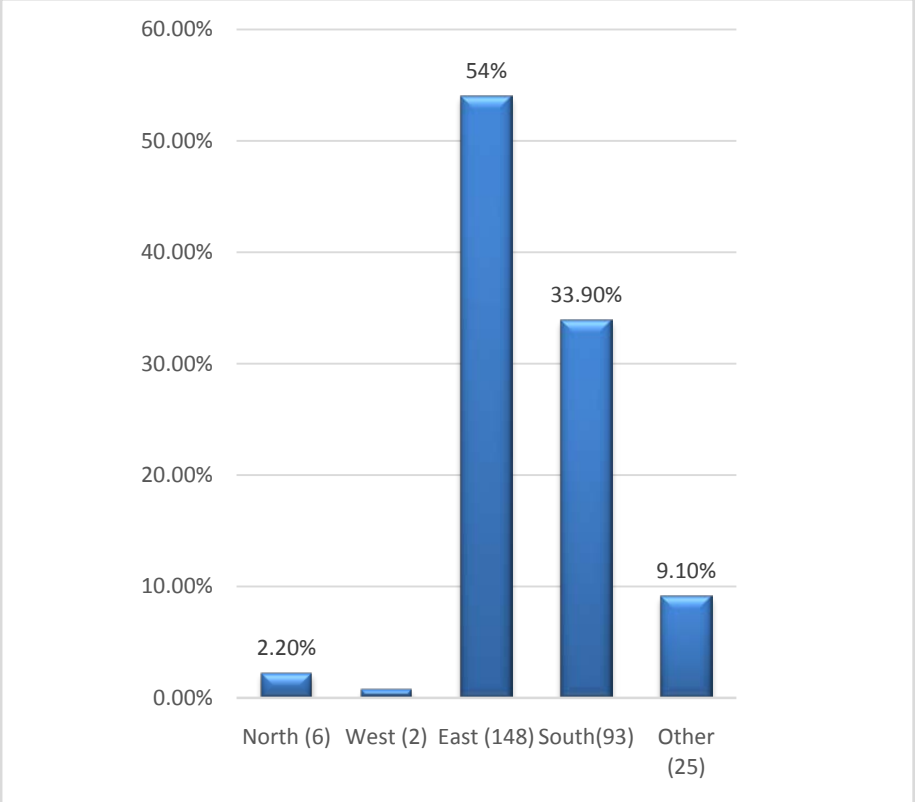


Figure 1. Geographical distribution of patients with lung adenocarcinoma

Majority patients were non smokers, comprising 117/165(70.9%) and 48(29.1%) smokers **(Figure 2)**

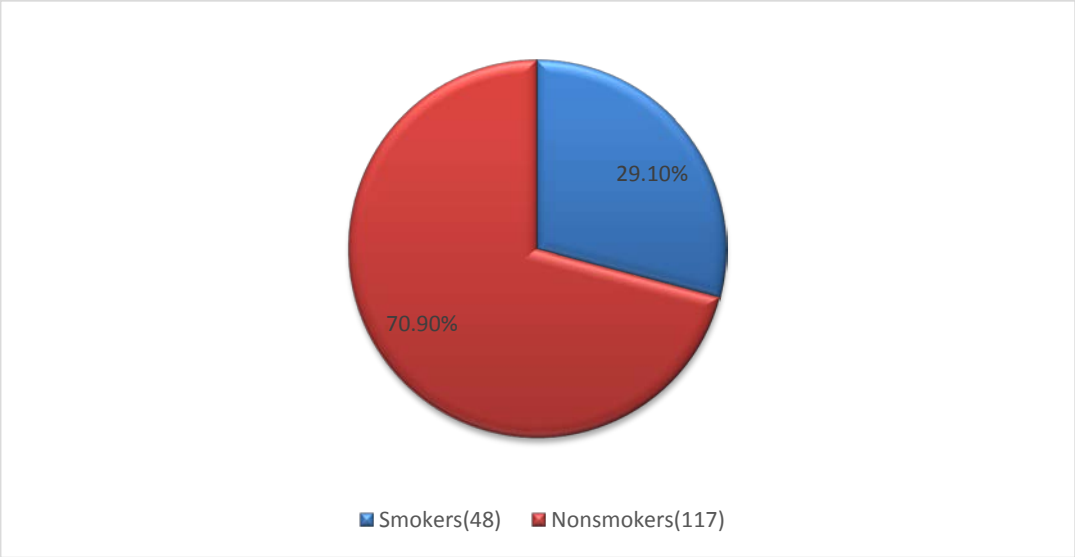


Figure 2. Distribution of smokers and non-smokers in lung adenocarcinoma

The commonest location of tumours was in the right lung 125 (45.6%), while 76(27.7%) were bilateral and 69(26.6%) were in the left lung. Although the right lung had a predilection for tumours in this study, the right upper and lower lobes were involved in equal frequencies 47/185(25.40%) and similarly there was no major difference between the left upper 31(16.8%) and left lower 35(18.9%) lobes in terms of distribution. The hilum was infrequently involved **(Figure 3)**

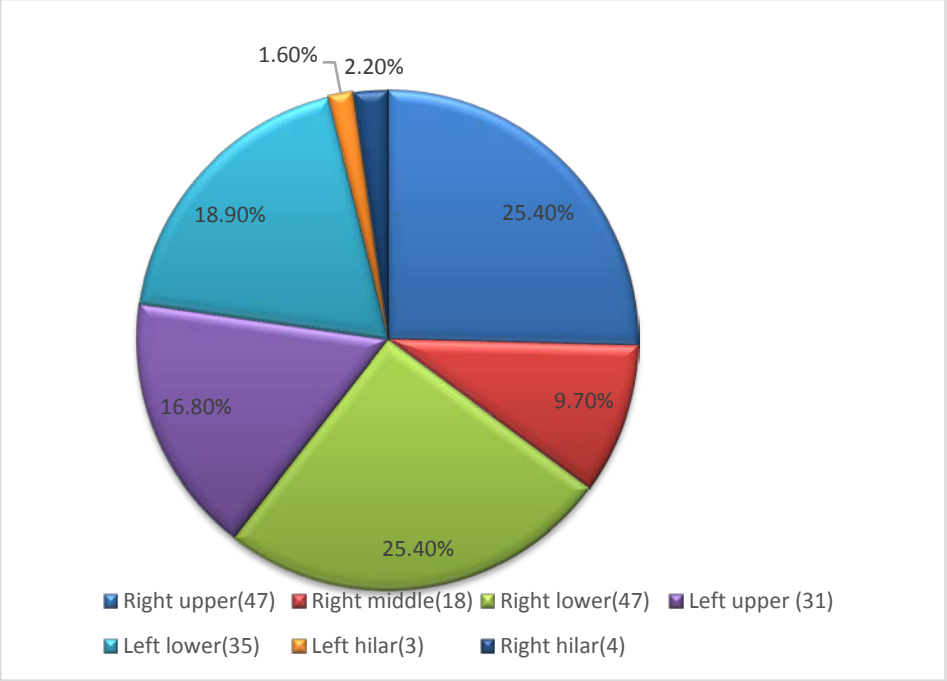


Figure 3. Distribution of lung adenocarcinomas in the lobes of lung.

Maximum tumour dimension was >3cm in 178/206 (86.4%) cases, documented on imaging (CT scan). A significant proportion of cases were stage IV 195/237 (82.2 %) at diagnosis, with fewer stage III 31/237 (13.1%) and 11/237 (4.7%) of Stage II **(Figure 4).** There were no cases of Stage I in this study. 216/232 (93.1%) had associated lymphadenopathy (P=0.633).

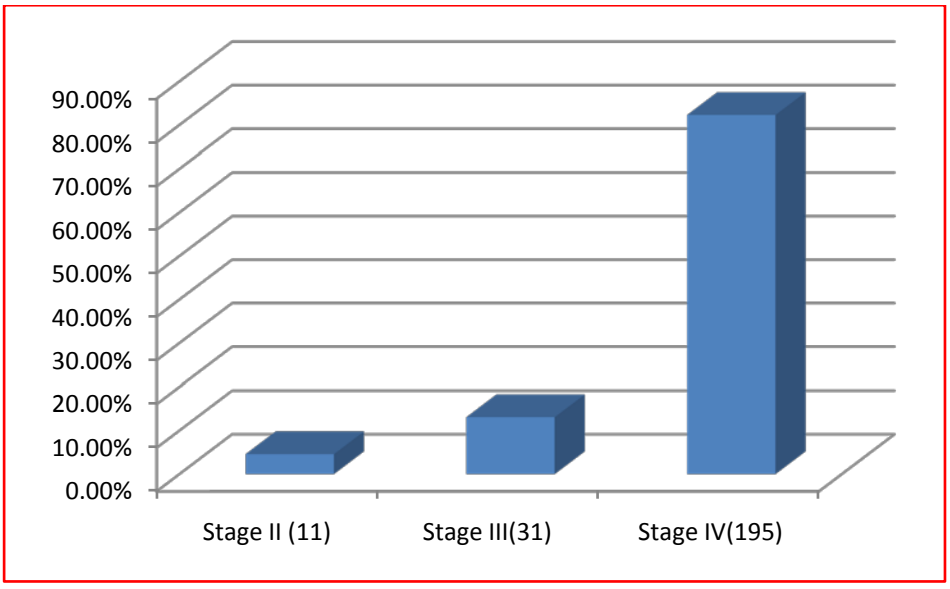


Figure 4. Distribution of stage of disease in lung adenocarcinoma.

The current IASLC/ATS/ETS classification(18,20) was used to categorize biopsies by the predominant histological subtype. Of 274 cases , there were acinar predominant adenocarcinoma 167 (61%) , 63(23%) solid with mucin production, 19 (7%) invasive mucinous adenocarcinomas, 11(4%)lepidic predominant and the lesser common subtypes being papillary 9 (3.30%) , two cases each of colloid (0.7%) and poorly differentiated, non-small cell carcinoma (NSCLC-NOS) (0.7%) and 1 micropapillary adenocarcinoma (0.3%) **(Figure 5).**

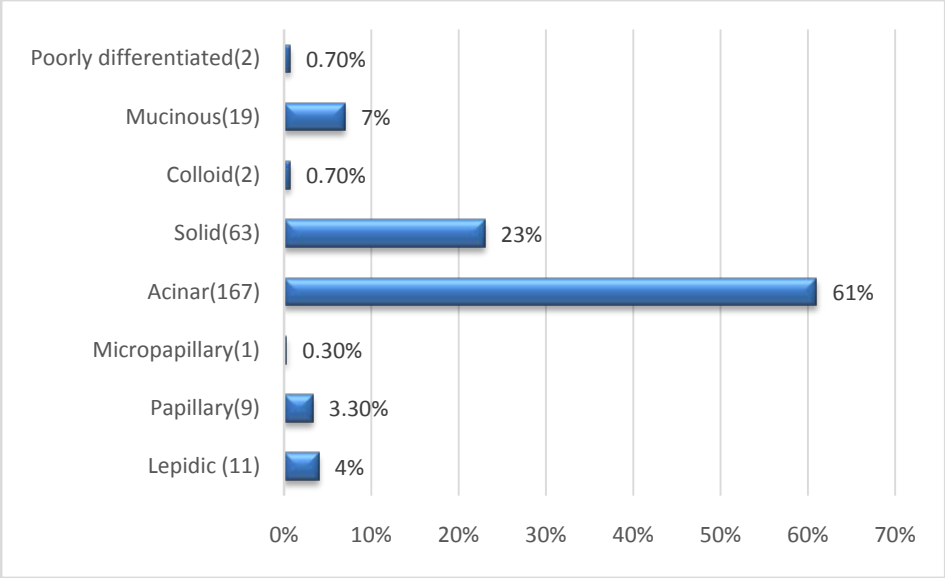


Figure 5. Distribution of predominant subtypes of adenocarcinoma.

Of 274 cases, the predominant cell type was polygonal 160(58.40%), followed by columnar 69(25.2%), cuboidal 38(13.9%), signet 4(1.4%), hobnail 2(0.7%) and clear cell 1(0.4%) **(Figure 6).**

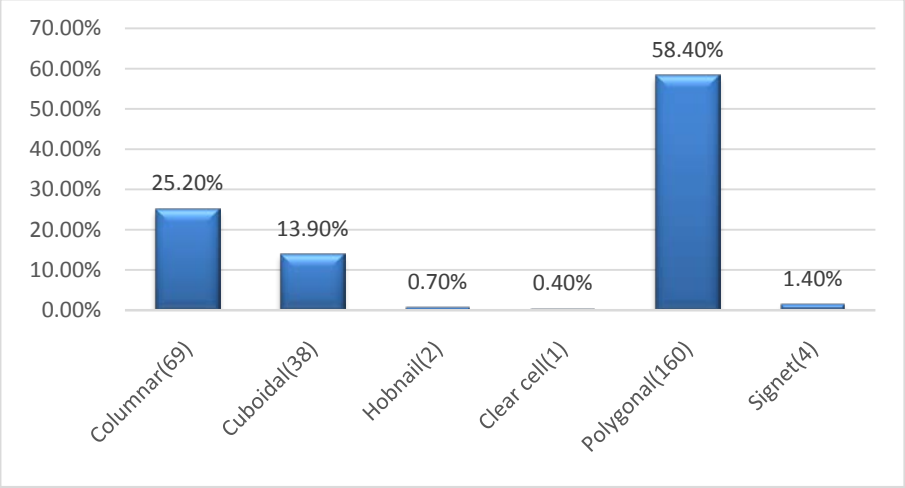


Figure 6. Distribution of predominant cell type in lung adenocarcinoma.

The papillary subtype was associated with columnar cells 7/9(77.8%) (P = 0.004), the solid subtype with polygonal cells 58(92%) (P=0.00), the invasive mucinous adenocarcinoma had predominantly columnar cells.15/19 (79%) (P=0.00) **(Table 1).**

Table 1. Association of cell type with predominant subtype

Cell type	N (%)	Columnar	Cuboidal	Hobnail	Clear cell	Polygonal	Signet	P value
Lepidic	11(4)	4(36.4)	2(18.2)	-	-	5(45.5)	-	
Papillar	9(3.3)	6(66.7)	1(11.1)	1(11.1)	-	**1(11.1)**	-	**0.003**
Micro-Papillar	1(0.3)	1(100)	-	-	-	-	-	
Acinar	167(61)	42(25.2)	31(18.6)	1(0.5)	-	93(55.7)	-	-
Solid	63(23)	-	**1(1.6)***	-	1(1.6)	**58(92)**	3(4.8)	**0.00** **0.001***
Mucinous	19(7)	**15(79)**	3(15.8)	-	-	**1(5.2)**	-	**0.00**
Colloid	2(0.7)	1(50)	-	-	-	1(50)	-	
NSCLC-NOS	2(0.7)	-	-	-	-	1(50)	1(50)	

P values that are significant are highlighted in the table

Grade of differentiation was mainly poorly differentiated 151(55.1%), moderately differentiated 115(42%) and well differentiated 08(2.9%) **(Figure 7)**.The papillary subtype was moderately differentiated 9/9(100%) (P=0.00) and the solid subtype was poorly differentiated 57/63(90.4%). In contrast, all the lepidic tumours were not well differentiated **(Table 2).**

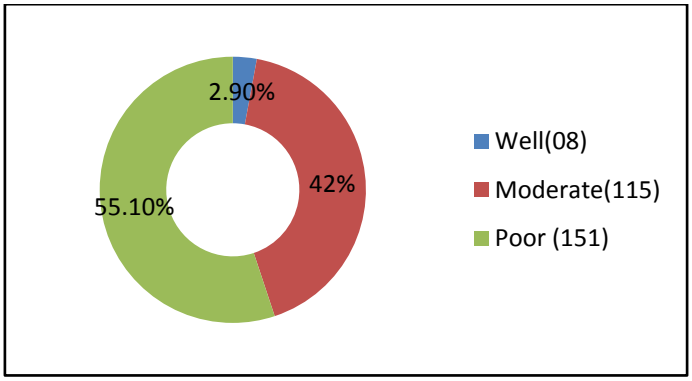


Figure 7. Association of grade of differentiation with predominant subtype.

Table 2. Association of Grade of differentiation with predominant subtypes.

		Grade of differentiation			
Subtypes	**N=274(%)**	**Well**	**Moderate**	**Poor**	**P value**
Lepidic	11(4)	03(27.3)	**05(45.5)**	03(27.3)	**0.002**
Papillary	09(3.3)	-	**9(100)**	-	**0.00**
Acinar	167(61)	4(2.4)	77(46.10)	86(51.5)	
Solid	63(23)	-	06(9.6)	**57(90.4)**	0.00
Mucinous	19(7)	1(5.3)	16(84.2)	02(10.5)	0.00
Micropapillary	01(0.3)	-	1(100)	-	-.
Colloid	02(0.7)	-	1(50)	1(50)	-
NSCLC-NOS	02(0.7)	-	-	2(100)	-

P values that are significant have been highlighted in the table

A significant percentage of tumours were associated with mucin production, desmoplasia, stromal elastosis, lymphovascular invasion and necrosis**. (Table 3)**

Table 3. Associated features with lung adenocarcinoma.

Associated features	N	Present(%)	Absent(%)	P value
Mucin production	274	226(82.5)	48(17.5)	0.002
Desmoplasia	274	267(97.4)	7(2.6)	0.016
Stromal elastosis	274	252(92)	22(8)	0.05
Lymphovascular invasion	274	227(82.8)	47(17.2)	0.013
Necrosis	274	175(63.9)	99(36.1)	0.180

Majority cases, 226/237(95.35%) were in Stage III/IV of disease. Lymphovascular invasion was associated with 187/226 (82.7%) cases in stage III/ stage IV disease **(Table 4).** However, a statistical significance was not established between presence of lymphovascular invasion and stage of disease (P=0.105) **(Table 5).**

Table 4. Association of stage of disease with predominant subtype

Subtypes	N =237(%)	Stage		
		II	III	IV
Lepidic	11(4.6)	1 (9.1)	1(9.1)	9(81.8)
Papillary	9(3.8)	0	0	9(100)
Acinar	143(60.4)	5(3.5)	17(11.9)	121(84.6)
Solid	55(23.2)	3(5.4)	11(20)	41(74.6)
Mucinous	16(6.8)	2(12.5)	3(18.8)	11(68.8)
Colloid	1(0.4)	0	0	1(100)
NSCLC-NOS	1(0.4)	0	0	1(100)
Micropapillary	1(0.4)	0	0	1(100)

Table 5. Correlation of lympho-vascular invasion with stage of disease.

Lymphovascular		Stage			P
invasion	N=237 (%)	II	III	IV	value
Present	195(82.3%)	8(72.7%)	30(93.8%)	157(80.9%)	0.105
Absent	42(17.7%)	3(27.3%)	2(6.3%)	37(19.1%)	

In relation to smoking status, the papillary 7/7 (100%), micropapillary 1/1 (100%), followed by the Lepidic predominant 4/5(80%) , acinar 75/102 (73.6%) and solid 20(55.6%) subtypes were frequent in non smokers. The solid subtype was relatively more frequent among smokers 20/36 (55.6%) (P=

0.05). However, there was no statistical significance established between smoking status and predominant subtype (Table 6).

Table 6. Association of smoking status with predominant subtype.

Subtypes	**N =**	**Smoker**	**Non**
Lepidic	5(3)	1(20)	4(80)
Papillary	7(4.3)	0	7(100)
Acinar	102(61.8)	27(26.4)	75(73.6)
Solid	36(21.9)	16(44.4)	20(55.6%)
Mucinous	13(7.8)	4(30.8%)	9(69.2%)
Micropapillary	1(0.6)	0	1(100%)
NSCLC-NOS	1(0.6)	1(100%)	0

The majority cases 189/274 (69%) had a chronic inflammatory response, 83(30.3%) with mixed inflammatory infiltrate and 2(0.7%) with acute inflammation **(Figure 8)** with no statistical significance between a particular inflammatory response and histological subtype **(Table 7).**

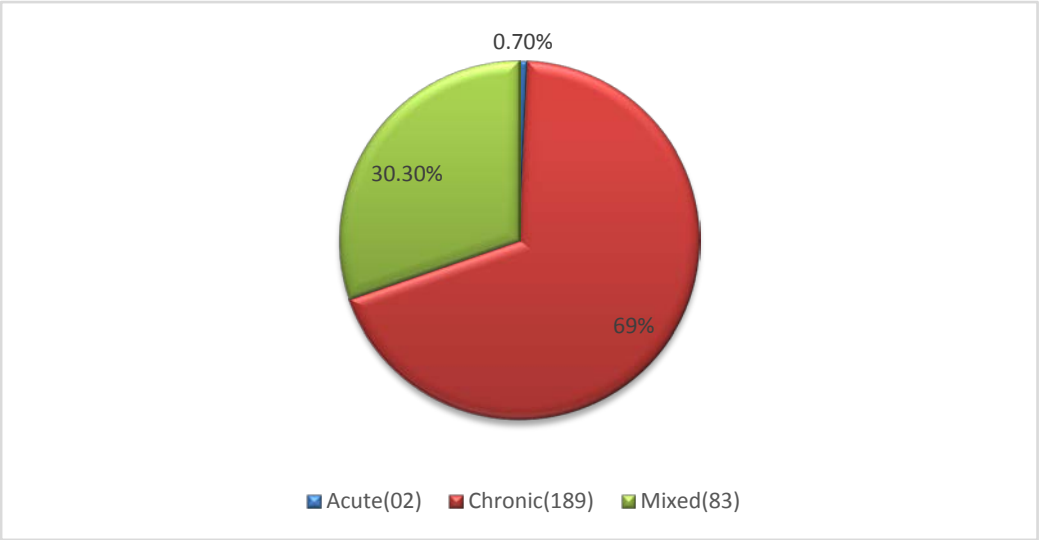


Figure 8. Distribution of inflammatory response in lung adenocarcinomas.

Table 7. Association of inflammatory response with predominant subtype

		Inflammation		
Subtypes	**N =274(%)**	**Acute**	**Chronic**	**Mixed**
Lepidic	11(4)	0	9(81.8)	02(18.2)
Papillary	09(3.3)	0	7(77.8)	2(22.2)
Acinar	167(61)	1(0.5)	111(66.5)	55(33)
Solid	63(23)	0	47(74.6)	16**(**25.4**)**
Mucinous	19(7)	0	13(68**)**	6(31.6)
Micropapillary	01(0.3)	0	1(100)	0
Colloid	2(0.7)	0	1(50)	1(50)
NSCLC-NOS	2(0.7)	1(50)	0	1(50)

The majority of tumours were TTF-1 positive 213/263(81%), including 90% lepidic predominant and the remaining less frequently, including two cases, each of colloid and poorly differentiated and one case of micropapillary carcinoma **(Figure 9 , Table 8).** There was no statistical significance between TTF-1 immunohistochemistry and predominant subtype.

Almost all cases were for positive for CK7 134/135(99.3%) and BerEp4 63/65 (97%). A minority of tumours were positive for CK20.

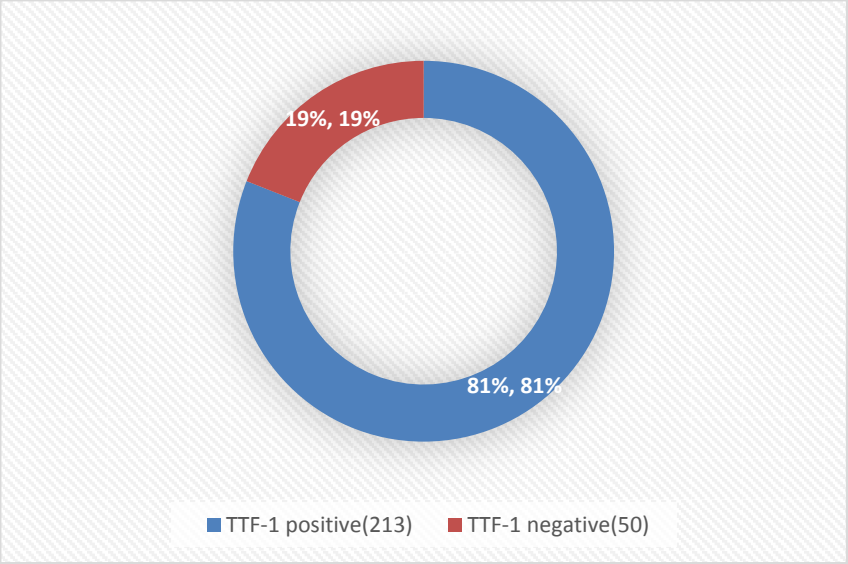


Figure 9. Distribution of TTF-1 immunohistochemistry in lung adenocarcinomas.

Table 8. Association of TTF-1 immunohistochemistry with predominant subtypes

Subtype	N =263(%)	TTF -1	
		Positive	Negative
Lepidic	10(3.8)	9(90%)	01(10%)
Papillary	09(3.4)	7(77.8)	2(22.2%)
Acinar	161(61.2)	134(83.2)	27(16.8)
Solid	60(22.8)	45(75)	15(25%)
Mucinous	18(6.8)	13(72.2%)	05(27.8%)
Micropapillary	1(0.4)	1(100%)	0
Colloid	2(0.8)	2(100%)	0
NSCLC-NOS	2(0.8)	2(100%)	0

In this study we had one case with hobnail cell type positive for TTF-1 (100%), followed by 89% of cuboidal cell type, 81% of polygonal cell type and 76% columnar cell type which were TTF-1 positive . There was no statistical significance established between cell type and TTF-1 immunohistochemistry (P= 0.65) **(Table 9).**

Table 9. Correlation of TTF-1 immunohistochemistry with predominant cell type

Cell type	N (%)	TTF1 positive	TTF-1 negative
Columnar	67	50 (76)	16(24)
Cuboidal	37	33 (89)	04 (11)
Hobnail	01	01 (100)	0
Clear cell	01	0	01 (100)
Polygonal	154	125(81)	29 (19)
Signet cell	03	03 (100)	0

Of 274 cases, majority presented in advanced stage of disease at diagnosis, only 6 tumours underwent resection. Amongst the 6 resection specimens, the invasive mucinous adenocarcinoma was the commonest subtype 3/6 (50%) 2 (33.3%) cases of acinar adenocarcinoma and 1 (16.7%) case of solid adenocarcinoma. These tumourswere more common in the left lung, majority being larger than 3cm in greater dimension (hence with radio-pathologic correlation a diagnosis of AIS, MIA was ruled out as these tumours had the largest dimension, >3cm. These tumours were in stage 2/3 of disease, in contrast to the small biopsy specimens , predominantly in stage 4 disease **(Table 10)**

Table 10.Clinico-pathological details in resection specimens

S. No	Final diagnosis	Site	Size	Stage
1.	Acinar	Left upper	<3cm	NA
2	Acinar	Right lower	>3cm	3
3	Mucinous	Left upper	>3cm	2
4	Solid	Right lower	>3cm	2
5	Mucinous	Left hilar	>3cm	3
6	Mucinous	Left lower	>3cm	2

On gross examination: All tumours were ill circumscribed, majority with infiltrative margins and presence of necrosis. Visceral pleural invasion was seen in 4/6 (66.6%) tumours. There was no gross evidence of haemorrhage **(Table 11).**

Table 11. Gross findings in resection specimens

S. No	Diagnosis	Circumscription	Infiltrative Margins	Pleural Invasion	Haemorrhage	Necrosis
1.	Acinar	Absent	Present	Present	Absent	Present
2.	Acinar	Absent	Present	Absent	Absent	Present
3.	Mucinous	Absent	Present	Present	Absent	Present
4.	Solid	Absent	Absent	Absent	Absent	Absent
5.	Mucinous	Absent	Present	Present	Absent	Present
6.	Mucinous	Absent	Present	Present	Absent	Present

EGFR mutational analysis was performed on a subset of 120 cases by PCR gene sequencing and 47 (40.9%) were found to harbour a mutation in any one of the four exons (18, 19, 20, 21) that are known to be hotspots of mutations, except one case which had a combined mutation. The majority 36(73.5%) were associated with exon 19 mutation with del E746-A750,as the commonest form and several other uncommon deletions **(Figure 10).** There were 12(24.5%) cases with exon 21 mutations, all with L858R gene type mutation. There was one case (2%) with a combined exon 20 (T790M) and 21 mutation (L858R) **(Figure 11).**

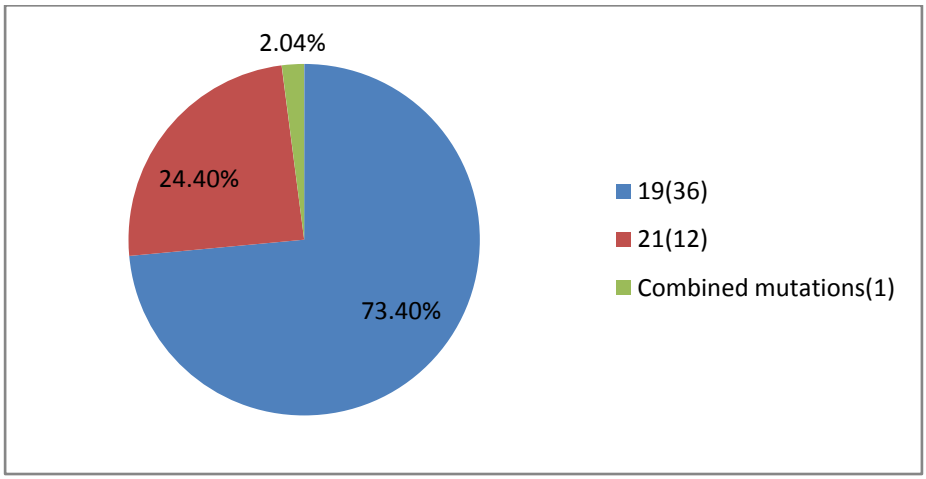


Figure 10. Distribution of EGFR mutations among lung adenocarcinoma (Exons 18-21)

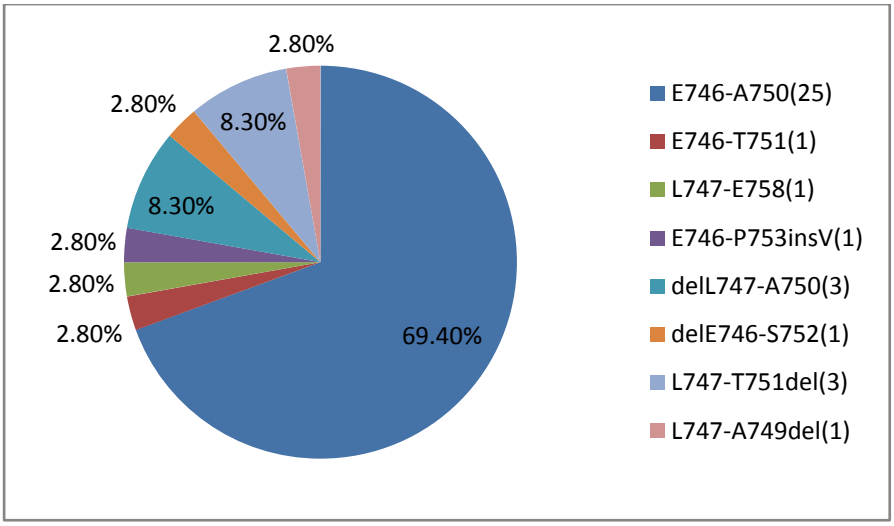
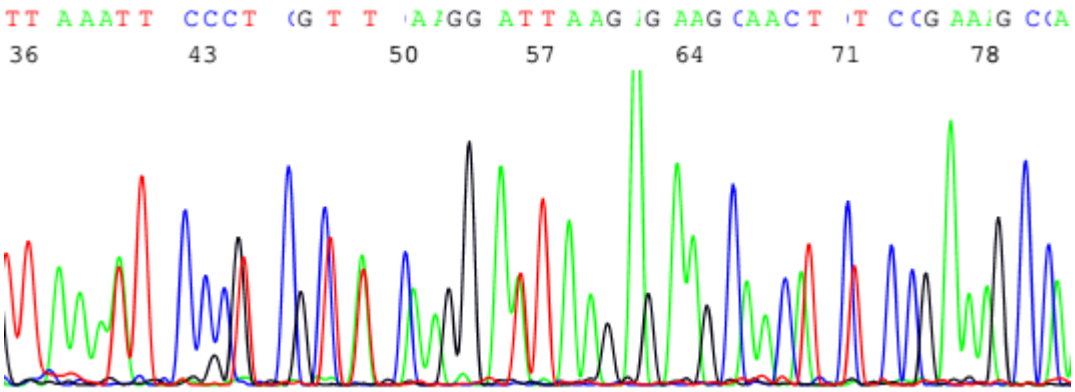


Figure 11. Spectrum of mutations in exon 19 of EGFR.

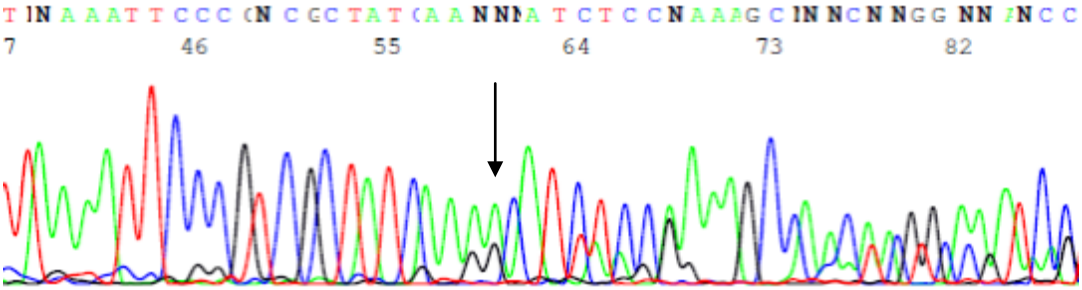
Table 12. Shows the prevalance of EGFR mutational status in Indian Studies, ranging from 25%to 51.8%. All studies showed predominance of exon 19 mutation followed by exon 21 mutations.

Table 12.Comparison of EGFR mutational status in Indian Studies.

Mutations	Present study	Bhat et al	Sahoo et al	Veldore et al	Jay Mehta	Doval et al	Chougule et al	Norhonha et al
N=	120	106	220	1036	367	166	1018	111
% mutation	40.9%	39.6%	51.8%	40.3%	32%	25.9%	25%	35%
Exon18	0%	2.4%	7.9%	4.5%	ND	2.3%	5.8%	2.5%
Exon 19	73.5%	76.2%	51.6%	61%	76%	51.2%	52.9%	74%
Exon 20	2%	4.8%	13.6%	4.1%	ND	4.7%	2.4%	0.5%
Exon 21	24.5%	16.6%	26.2%	31.8%	24%	34.9%	38%	23%

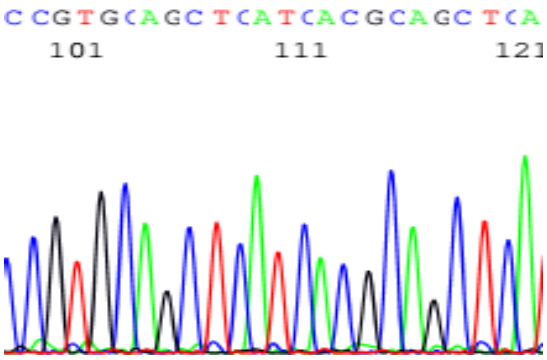


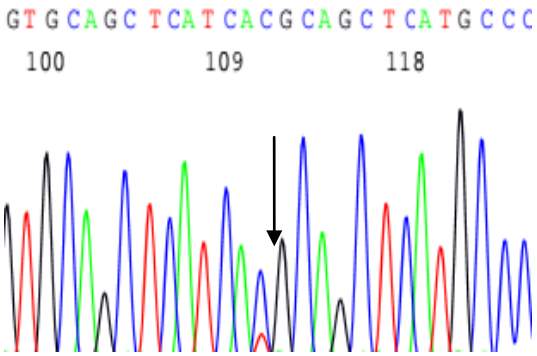
Wild Type Exon 19



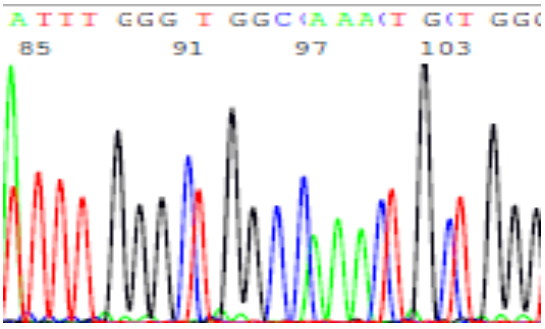
Exon 19- E746-A750 del

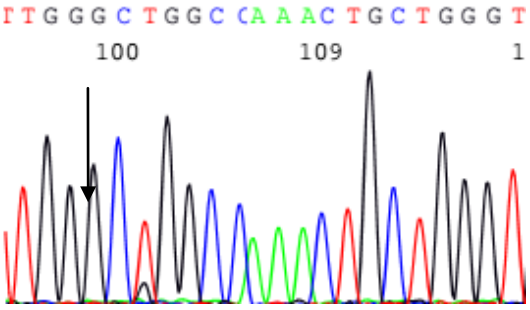
Figure 12. Exon 19 wild type and with E746-A50 deletion





EXON 20- Wild Type **EXON 20- T790M**





EXON 21- Wild type **EXON 21- L858R**

Figure 13. Combined mutation in exon 20(T790M) and exon 21 (L858R)

There was no statistical association of EGFR mutational status with geographical distribution amongst patients in this study. EGFR mutations were more common among women(48.1%) than in (32.4%) in men and also more prevalent in non smokers(45.5%) than smokers (27.8%). TTF1 positivity was significantly associated with EGFR mutational status (P= 0.007). Regression analysis of patients with mutation and TTF1 positivity showed a relative risk of 4.995 **(Table 13).**

Table 13. Correlation of EGFR mutational status with clinico-pathological features and TTF-1 immunohistochemistry.

Characteristics	**Cases examined N=120 (%)**	**EGFR mutations**		**P value**
		Wild type	**Mutated**	
Location				
North	04 (3.3%)	03(75%)	01(25%)	0.777
West	2 (1.7%)	01(50%)	01(50%)	
East	45(37.5%)	30(66.7%)	15(33.3%)	
South	61(50.8%)	34(55.7%)	27(44.3%)	
Outside India	8(6.7%)	05(62.5%)	03(37.5%)	
Gender				
Male	68(56.7%)	46(67.6%)	22(32.4%)	0.08
Female	52(43.3%)	27(51.9%)	25(48.1%)	

Characteristics	Cases examined N=120 (%)	EGFR mutations		P value
		Wild type	Mutated	
Smoking Status	N=84 (%)			
Smoker	18(21.4%)	13(72.2%)	05(27.8%)	0.517
Non Smoker	66(78.6%)	36(54.5%)	30(45.5%)	
TTF-1	N=112(%)			**0.01**
Positive	96(85.7%)	52(78.8%)	44(45.8%)	
Negative	16 (14.3%)	14(21.2%)	02(4.3%)	

A comparison of the mutational pattern with the histological subtype indicated that mutations were most commonly seen in Lepidic subtype 3/5 (60%), followed by Papillary 4/9 (44.4%), Acinar 29/68 (42.7%) and 10/24 Solid (41.7%) subtypes in decreasing frequencies. Invasive mucinous tumours were infrequently associated with EGFR mutations (P= 0.015) **(Table 14)**

Table 14. Comparison of EGFR mutations with predominant subtype.

Subtype	N= 120 (%)	EGFR mutations		P
		Wild type	Mutated	value
Lepidic	05	2(40%)	3(60%)	0.380
Papillary	09	5(55.6%)	4(44.4%)	0.737
Acinar	68	39(57.3%)	29(42.7%)	0.343
Solid	24	14(58.3%)	10(41.7%)	1.000
Mucinous	13	12(92.3%)	1(7.7%)	0.015
Micropapillary	01	1(100%)	-	-

There was no significant statistical association of age, gender, smoking status and TTF-1 postivity when compared exclusively with exon 19 and exon 21 mutational statuses (Table 15)

Table 15. Comparison of clinico-pathological features between cases with exon 19 and 21 mutations .

Characteristics	N(%)	Exon 19	Exon 21	P value
Age	37	57.7±13.3	61.36±8.07	0.411
Male	23	18(78.3)	05(21.7)	0.852
Female	24	19(76)	06(24)	
Smoker	05	04(80)	1(20)	0.676
Non smoker	31	22(71)	09(29)	
TTF-1 positive	45	34(75.6)	11(24.4)	0.424
TTF-1 negative	02	2(100)	-	

Hypothesis test summary for the above comparison

Null Hypothesis	Test	Sig	Decision
The distribution of age is the same across categories for exons	Independent-samples Mann-Whitney U test	0.411	Retain null hypothesis.

We also compared the predominant cell type with EGFR mutations, smoking status, grade and stage. Although the polygonal cell type (44.6%) was most frequently associated with EGFR mutations, the cuboidal cell type was significantly associated with EGFR mutations (P=0.013). There was only one case of hobnail cell type with an EGFR mutation. The polygonal cell type were associated with poor differentiation (86.1%) (P=0.00) and also with stage4 disease (82.01%) **(Table 16).**

Table 16. Association of predominant cell type with EGFR mutational status and clinico-pathological features.

Char.	N (%)	Columnar	Cuboidal	Hobnail	Clear	Polygonal	Signet	P* value
EGFR								
Wild	73	26(35.7)	**05(6.8)**	0	0	42(57.5)	0	**0.013**
Mutated	47	14(29.8)	**11(23.5)**	1(2.1)	0	21(44.6)	0	-
Smoker	48	11(23)	08(16.6)	0	1	27(60.4)	1	-
Non smoker	117	36(30.8)	15(12.8)	1(0.8)	0	65(55.6)	0	

Stage 2	11	03(7.1)	01(9)	0	0	07(63.6)	0	
Stage3	31	08(25.8)	05(16.1)	0	0	18(58.1)	0	
Stage 4	195	51(26.2)	28(14.3)	1(0.5)	1(0.5)	112(57.4)	2	
Grade								
Well	08	**06(75)**	01(12.5)	0	0	01(12.5)	0	**0.004**
Moderate	115	54(47)	29(25.3)	1(0.8)	0	30(26.1)		
Poor	151	10(6.7)	08(5.3)	0	1(0.6)	**130(86.1)**	2(1.3)	**0.00**

*P values that are significant have been highlighted in the table.

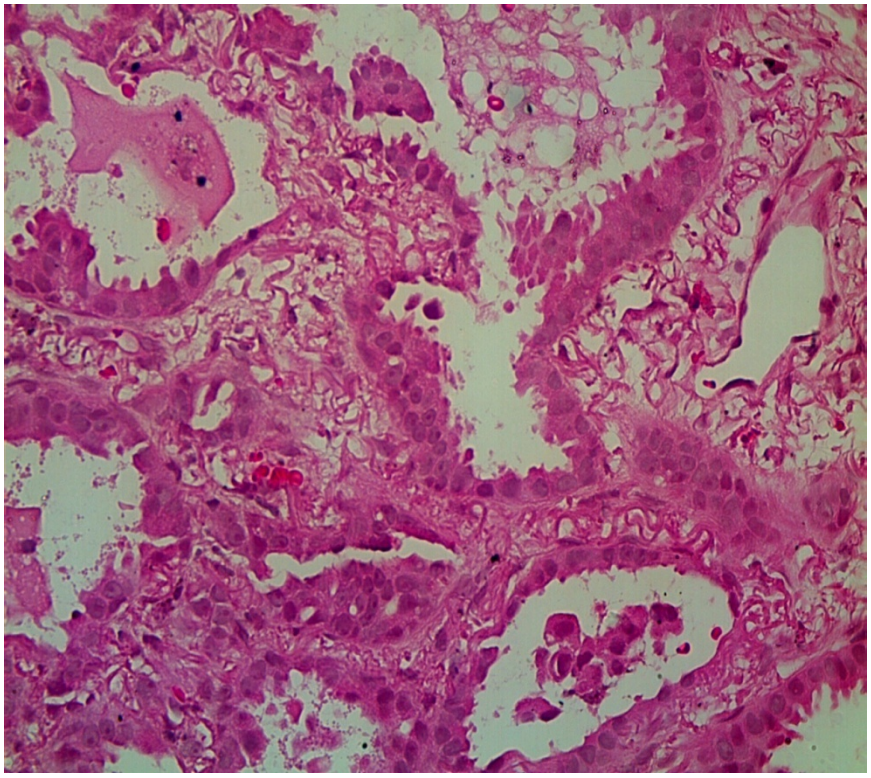


Fig 14. Acinar adenocarcinoma with hobnail and elastosis (H&E 400x)

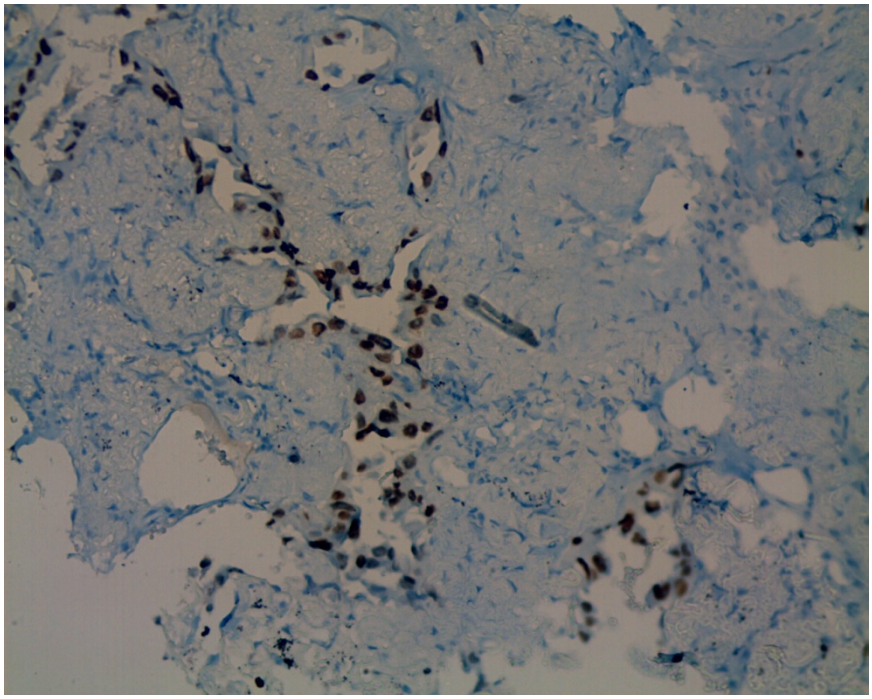


Fig 15. TTF-1 positive acinar adenocarcinoma (200x)

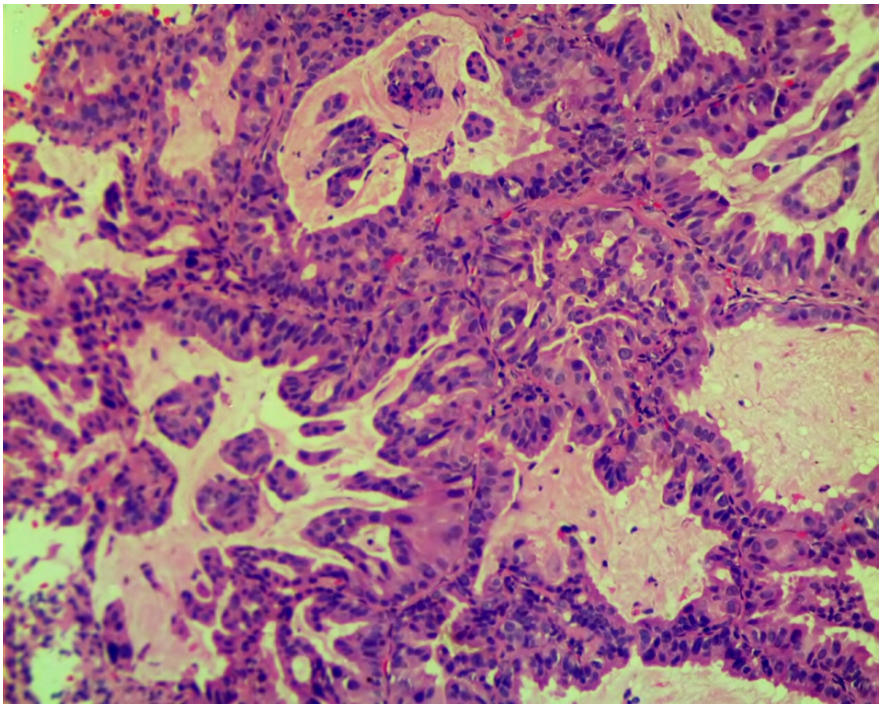


Figure 16. Complex acinar-papillary structures (H&E, 200X)

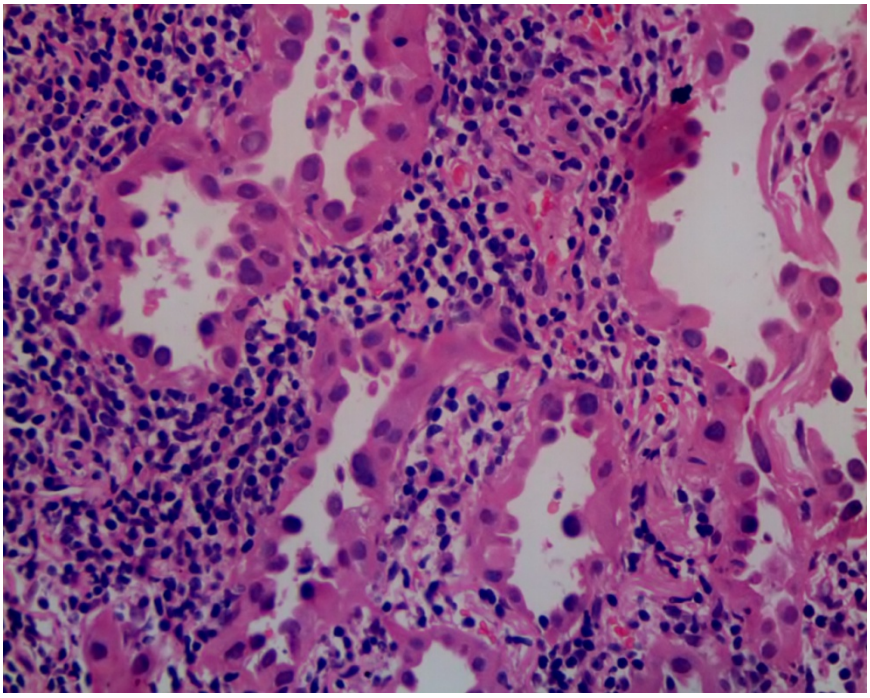


Figure 17. Acinar adenocarcinoma with lympho-plasmacytic infiltrates.

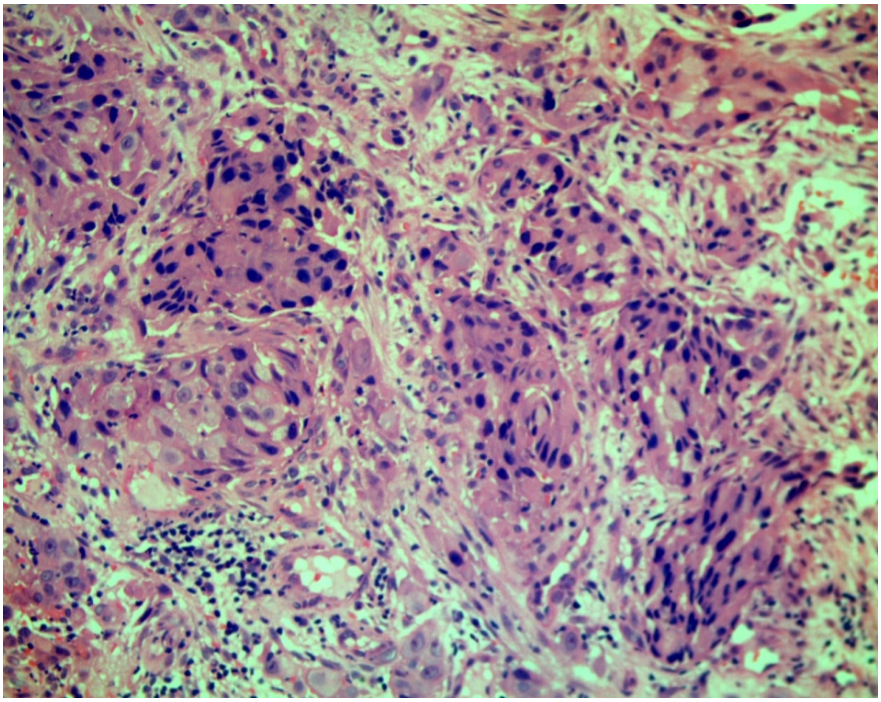


Figure 18. Solid adenocarcinoma (H&E 200X)

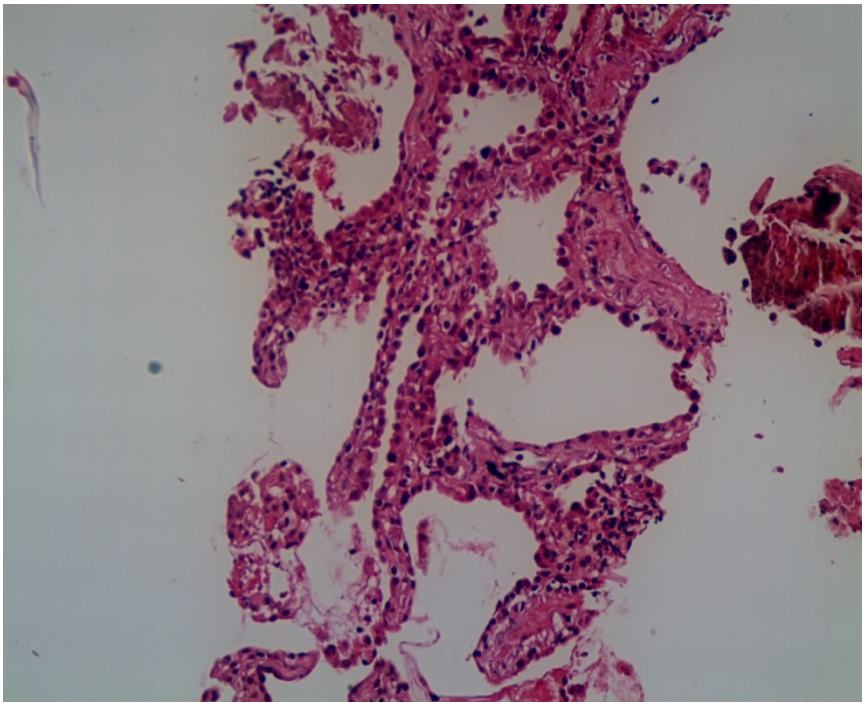


Figure 19. Non mucinous lepidic predominant adenocarcinoma (H&E, 200X)

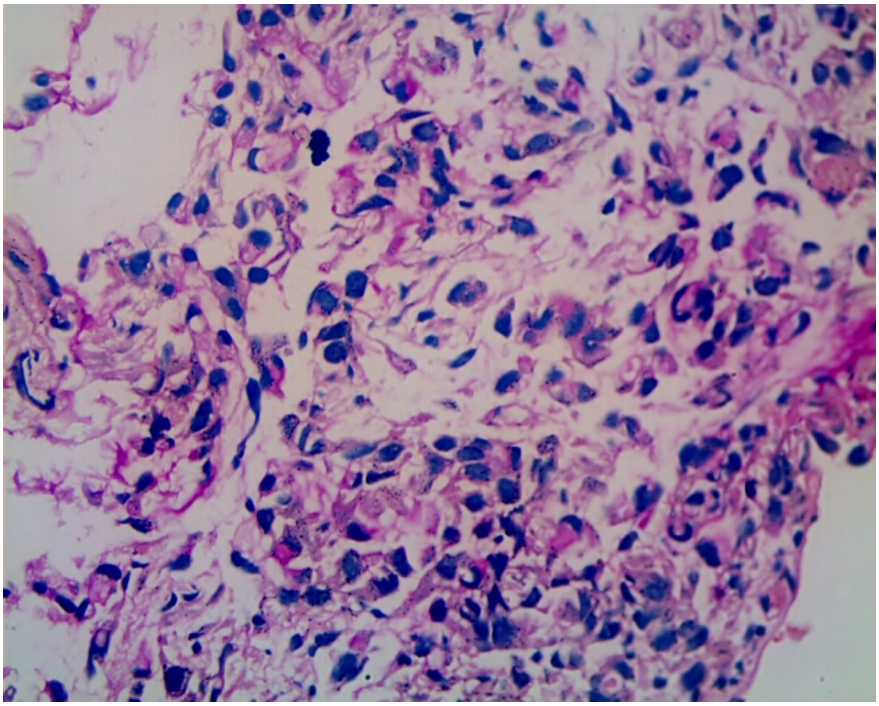


Figure 20. Adenocarcinoma with signet cell features (H&E, 400X).

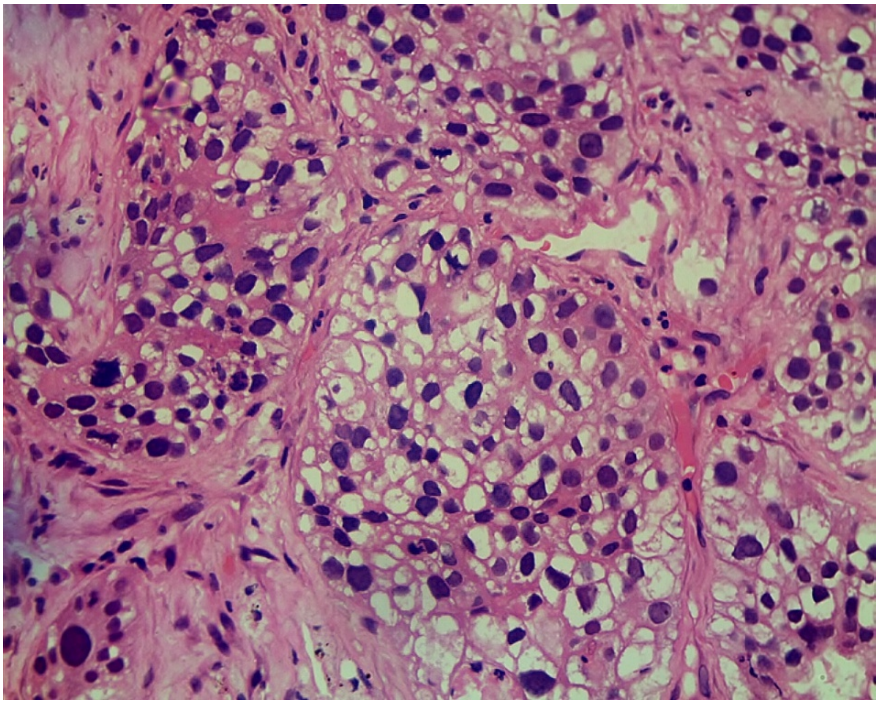


Figure 21. Adenocarcinoma with clear cell features (H&E, 400X)

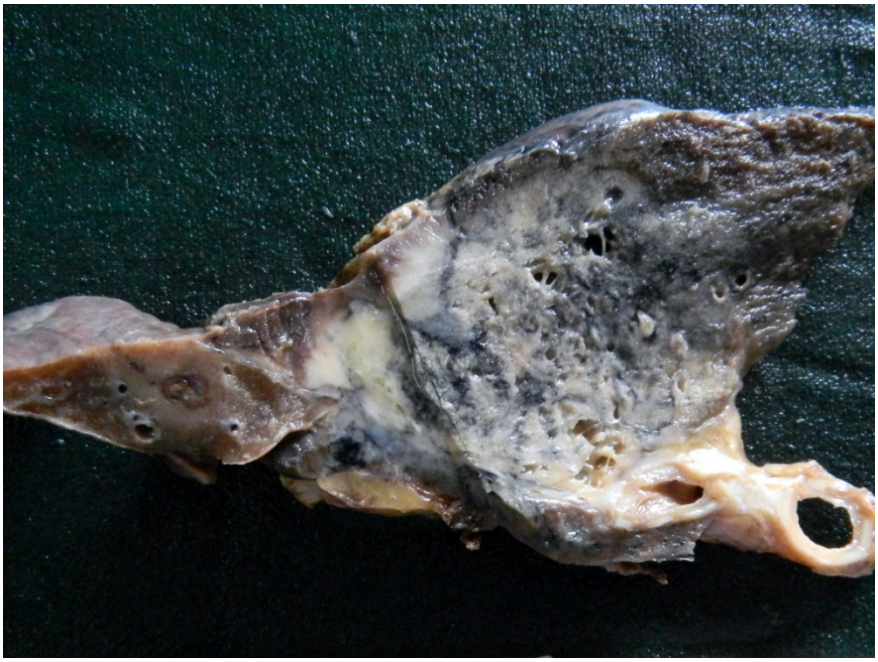


Figure 22. Gross specimen, invasive mucinous adenocarcinoma

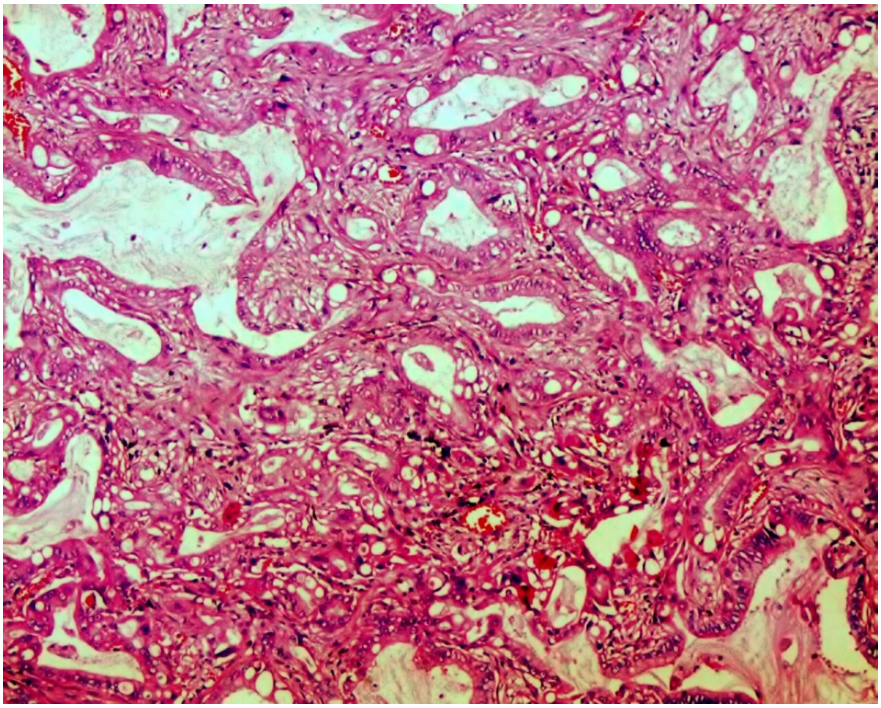


Figure 23. Invasive mucinous adenocarcinoma (H&E, 200X)

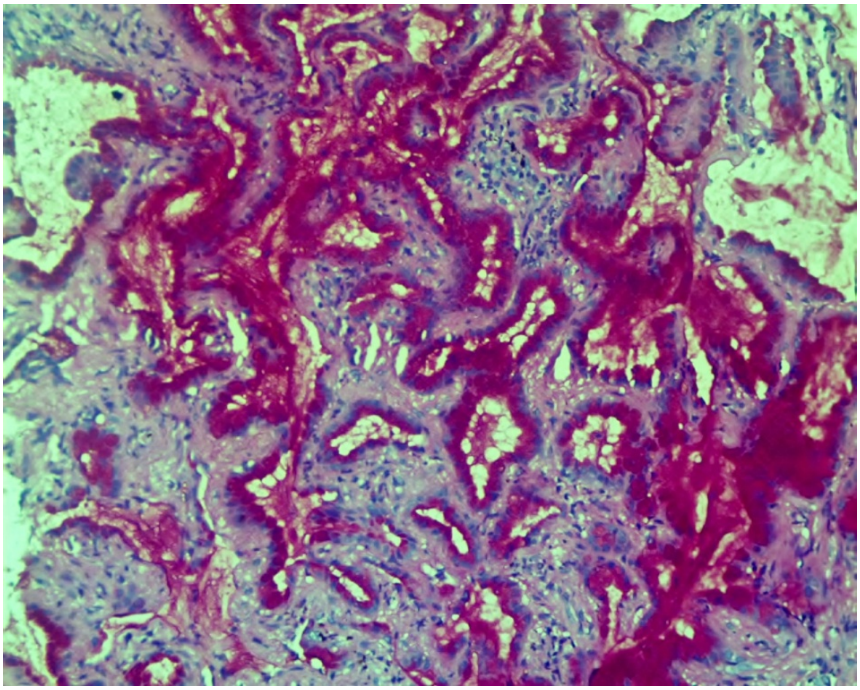


Figure 24. Invasive mucinous adenocarcinoma (PASD 200X).

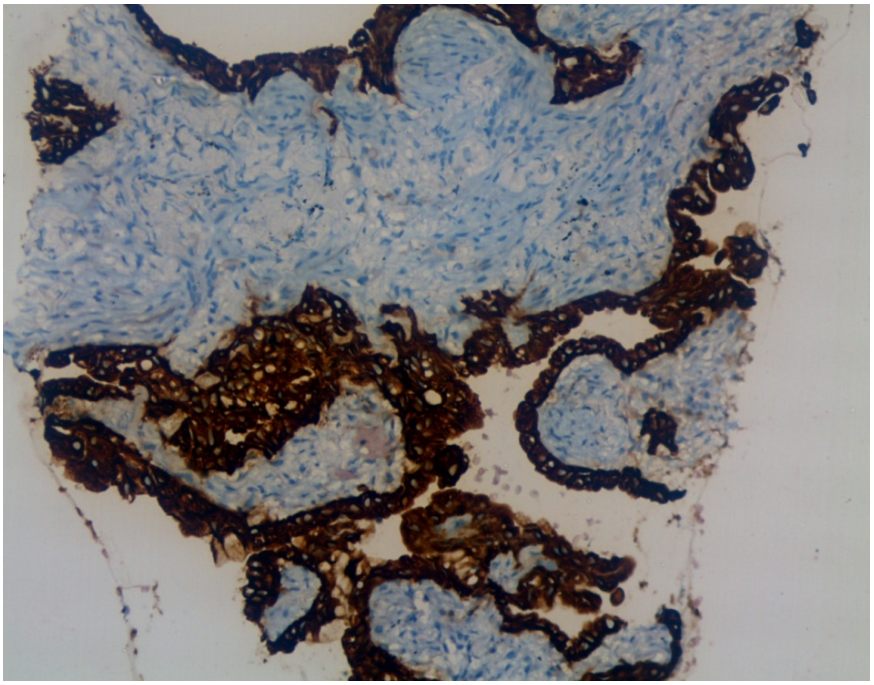


Figure 25. CK 7 positive, invasive mucinous adenocarcinoma (200X)

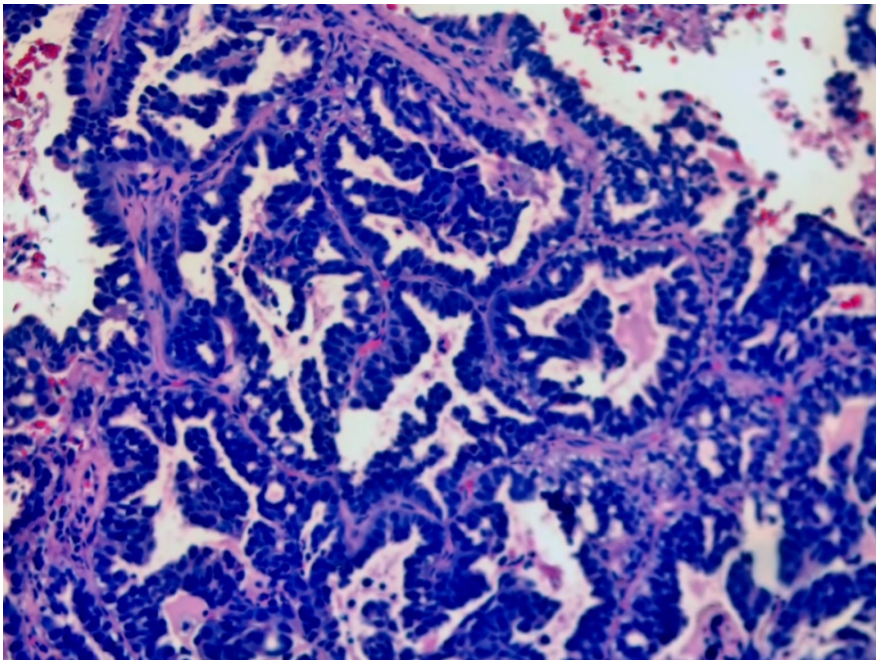


Figure 26. Papillary adenocarcinoma (H&E, 200X)

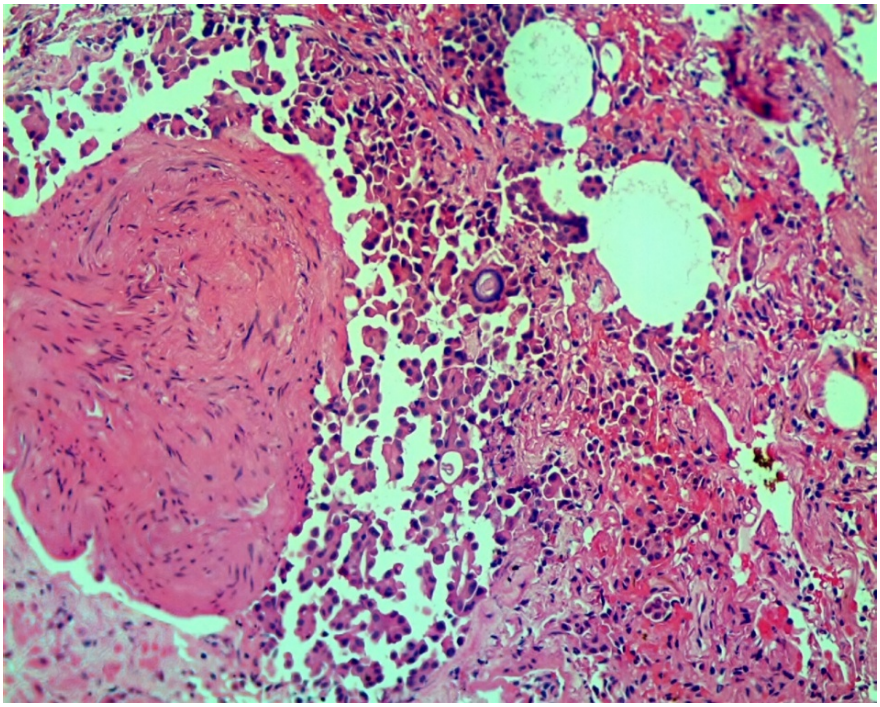


Figure 27. Micropapillary adenocarcinoma (H&E, 200X)

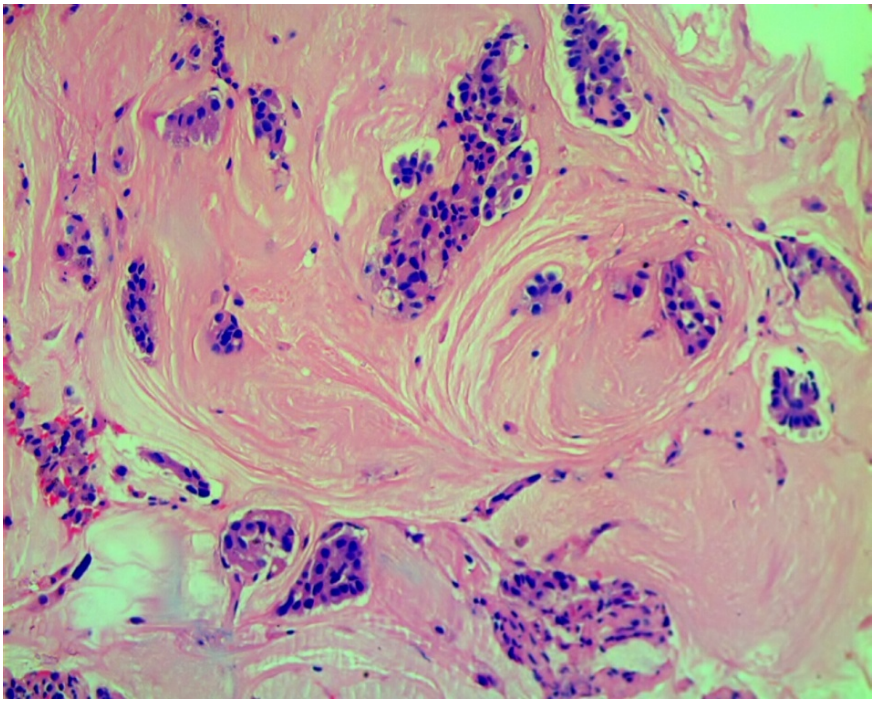


Figure 28. Colloid adenocarcinoma (H&E, 200X)

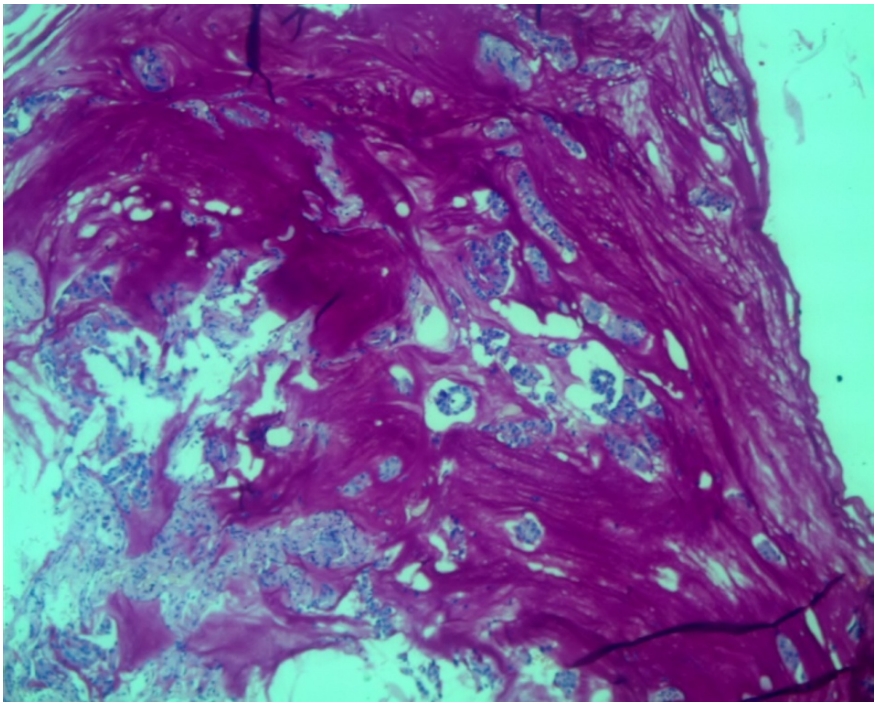


Figure 29. Colloid adenocarcinoma (PASD, 200X)

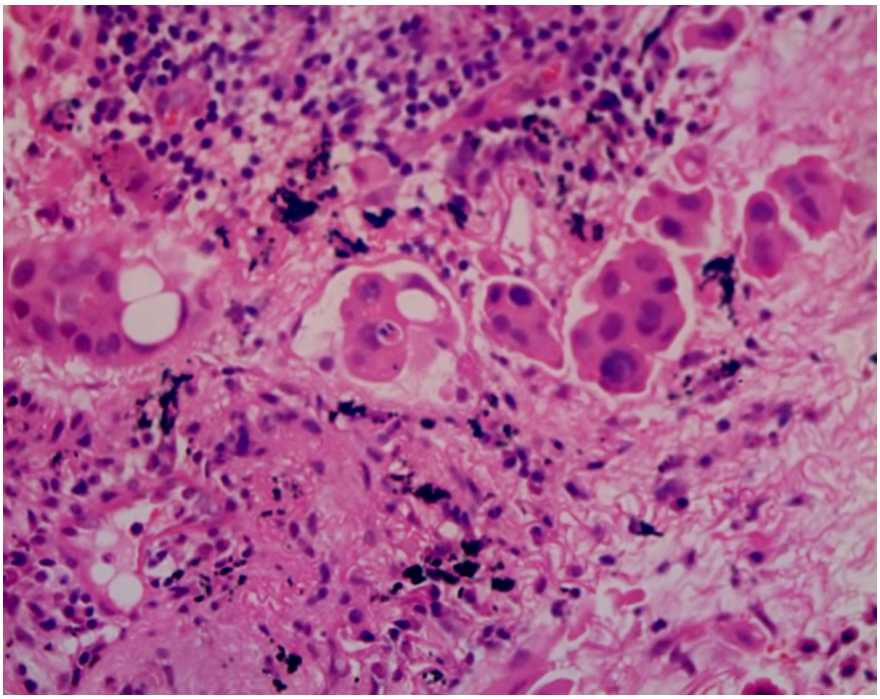


Figure 30. Lymphovasular invasion (H&E, 400X)

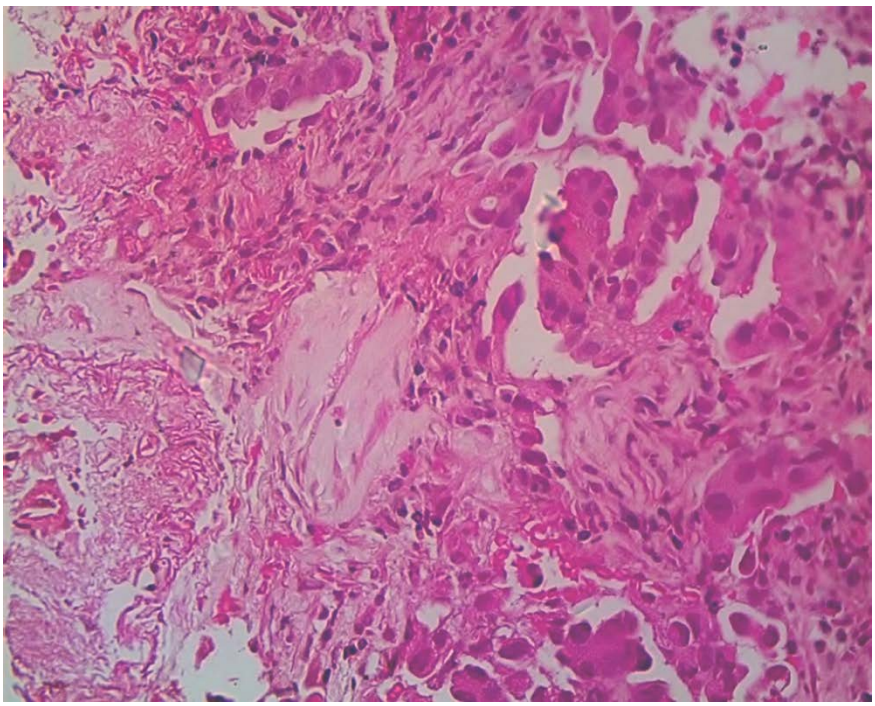


Figure 31. Stromal elastosis (H&E, 400X)

Discussion:

The prevalence of EGFR mutations in this study was 40.9% similar to a recent published study from our institute, Christian Medical College, Vellore, a tertiary care hospital in Southern India where a prevalence of 39.6% of EGFR mutations was reported, with favourable progression free survival response with chemotherapy followed by tyrosine kinase inhibitors (9). The prevalence of EGFR mutations in this study was similar to a study by Veldore et al in an Indian cohort, who reported a prevalence of 40.3% (99). Several other studies from India have reported prevalence of EGFR mutations ranging from 25%- 40.3%(55,74,100,101). A study by Sahoo et al in India, reported a slightly higher prevalence of EGFR mutations of 51.2%, possibly due to difference in methodology as this study used ARMS PCR, a more sensitive PCR sequencing method, than that of the present study. (10). The prevalence of EGFR mutation data from East Asian population ranges from 24% to 66.3% as EGFR mutations, being more common amongst East Asians (11-13) .

There was a predominance of exon 19 mutations, in our analysis with Del E746-A750 as the commonest mutation(69.4%) and 24.4 % exon 21 mutations, according to literature where small in-frame deletions in exon 19 and a point mutation (CTG to CGG) in exon 21 make up for ~90% of all mutations(3,65). We had a predominance of exon 19 mutations a subset likely to respond to tyrosine kinase inhibitors, according to a study by Jackman et al who found that EGFR exon 19 deletions have a longer survival with gefitinib

or erlotinib as compared with those having the L858R mutation which is an exon 21 deletion. (14).

There were no cases with exon 18 or 20 mutation; these point mutations have been described in 5% of the population with non-small cell carcinoma. Many other mutations are also described at low frequencies, but their relevance is not yet known(66) . There was one case with combined exon 20 (T790M) and exon 21 (L858R) mutations on treatment with gefitinib, the exon 20 (T790M) being probably "acquired resistance" after treatment with gefitinib. It is well documented in literature that in approximately half of the cases, tumour cells obtained after disease progression contained a second site mutation in the EGFR kinase domain, the most common >90% lesion involving C→T change at nucleotide 2369 in exon 20 that substitutes methionine for threonine at position 790 (T790M)(90)**.** The present study did not find any significant association of exon 19, 21 with age, gender, location, smoking status and TTF1.

EGFR mutations were significantly more common in tumours expressing TTF-1, hence TTF-1 positivity can be used as a predictive marker for EGFR mutations as published in several studies (15, 16) (17). By regression analysis, we found that patients that are positive for TTF-1 were 4.995 times more likely to have an EGFR mutation. However, with this classification there was no statistical significance established of any particular subtype with TTF1 in our study.

In this study, EGFR mutations were most commonly seen with lepidic predominant (60%), followed by papillary adenocarcinomas (44.4%). Literature has well documented the association of lepidic predominant adenocarcinomas with EGFR mutations (15, 18). In a study by Ping Li et al papillary adenocarcinomas were significantly associated with EGFR mutations(102). We had cases both of lepidic and papillary adenocarcinoma, predominantly associated with EGFR mutations. These observations are probably explained by the "Progression model", whereby it is hypothesised that the non-invasive lepidic subtype, would have progressed to the invasive pattern, commonly being papillary(28). A study by Motoi et al also showed that papillary and Micropapillary adenocarcinomas were frequently associated with EGFR mutations (17). However, we had only one case of Micropapillary adenocarcinoma which was associated with 'Wild' type mutations. EGFR mutations were infrequent in our patients with invasive mucinous adenocarcinomas as mucinous adenocarcinomas are commonly seen in smokers and are associated with KRAS mutations and rarely associated with EGFR mutations (19-21)

We compared the predominant cell type with EGFR mutations, smoking status, grade and stage. The Cuboidal cell type was significantly associated with EGFR mutations (68.6%)(P= 0.013). Only one case with hobnail predominant subtype was associated with EGFR mutations. The polygonal cell type was the most common amongst smokers (60.4%) as well as non- smokers (55.6%), with no statistical significance. The polygonal cell type was also associated with grade 3 (86.1%). (P=0.00). These findings were

reported in a study by Okada et al who proposed a five cell type classification, including the hobnail, polygonal, goblet, columnar/Cuboidal and mixed cell types and found that it was the hobnail type which was significantly associated with EGFR mutations($P<0.001$), followed by mixed, columnar/ Cuboidal, polygonal and goblet cells. The percentage of smokers was significantly higher amongst the Cuboidal/ columnar and polygonal cells in contrast to the hobnail and the mixed cell types (22).

The five cell types (hobnail, Cuboidal/ columnar, polygonal and mixed subtype) were compared with TTF-1 in a study by Okada et al (103)and found thatthe hobnail cell type was consistently associated with TTF-1 positivity in almost all cases (99%) followed by the mixed cell type (89%) , the Cuboidal/columnar cell type (54%) and the polygonal cell type (50%). Considering this outcome, they developed a hypothesis that, the carcinoma cells imitate characteristics of progenitor cells i.e. almost all hobnail cells develop at the terminal respiratory unit (TRU) while the mixed subtype arises more distal to than that of the terminal respiratory unit and the remainder (Cuboidal/columnar/polygonal) develop at the junction of TTF-1 positive and negative bronchioles, bronchi and bronchial glands. We had one case with hobnail cell type which was TTF1 positive. The majority 81% cases were TTF-1 positive across all cell types, TTF-1 being the best pneumocytic marker.

Invasion has been defined as presence of either of acinar, papillary, micropapillary patterns or myofibroblastic stroma that is associated with

invasive tumour/ the tumour displays lympho-vascular invasion, invades pleura or contains tumour necrosis.(19) All cases in this study were associated with an invasive component, 97.4% of these with a desmoplastic response and 92 % with stromal elastosis. According to a study by Xu et al "several types of invasion were identified i) invasion with areas of destruction of the alveolar pattern with relatively uniform acinar structures and without a desmoplastic response. ii) Invasion of orderly acini with associated desmoplasia comprising of reactive fibroblasts. iii) invasion with a desmoplastic response, compressed acinar structures or single tumour cells iv) Elastosis may occur in areas of lepidic spread, being prominent in central scar without invasive glands" (41) However, Noguchi et al in his study "separated alveolar collapse with elastosis from invasion with fibroblast proliferation, emphasizing the good prognosis associated with the former"(42) . The Noguchi classification reported that the absence of invasion imparted a benign prognosis to the tumour.

Based on the latest IASLC/ATS/ERS classification(18,20), it is the size of invasive component which is a cut off between low grade and intermediate grade tumours. Tumours with less than 5 mm invasive component are considered well differentiated neoplasm's and behave like non-invasive pure BAC's, recently described as AIS and MIA. Several studies have suggested that the invasive tumour component is an independent prognostic factor, and it could be a better predictor of prognosis than overall tumour size in lepidic predominant tumours(43)(44). Travis et al in their study concluded that the invasive tumour component which is vital for measurement rather than

the entire tumour size with non-invasive component, thus stating if this finding could impact the 'T' in the next TNM classification. XU et al(45) also stated that it is not the amount of invasion but the type of invasion which determines prognosis. In their study of lepidic predominant adenocarcinomas, they found that tumours in the absence of lymph vascular invasion, solid or single cell invasion had a good prognosis inspite of a large invasive area, including an elastotic scar. Invasion with desmoplasia with compressed and single cell growth is associated with worse prognosis compared with other patterns of invasion(45).

Majority were males(67.8%), (70.8%) were in stage IV and 55.1% of these were poorly differentiated with impeding survival. Lymphovascular invasion was in 82.8 % and 63.9% had areas of necrosis in similar to a study by Yoshizawa et al where higher stage, male gender, necrosis, poor differentiation and vascular invasion all were associated with decreased disease-free survival. (23).

Although the majority of patients in this study were non-smokers, adenocarcinomas, including the solid subtype were reported among smokers with a relatively high frequency and majority were poorly differentiated. Motoi et al had found that solid predominant adenocarcinomas had a strong correlation with high grade, poor differentiation and were associated with a worse survival of 34% as compared to 94% for moderate and well differentiated tumours($P<0.001$) and were associated with poor prognosis in

comparison to the non-solid subtype(P=0.001) (17). The solid adenocarcinomas are known to be associated with KRAS mutations(3).

In this study, papillary tumours were all moderately differentiated and the solid variant was poorly differentiated(P=0.00). According to a study by Yoshizawa,(51) tumour grade as per the WHO 2004 classification (well differentiated, moderately differentiated and poorly differentiated) were predictors of overall survival. Survival rates have been strongly correlated with stage and grade by Motoi et al (17) However, in the present study we did not find a significant comparison between the other grades and stages.

Thus, the new IASLC/ ATS/ERS classification identifies histologic subtypes/ cell types and its correlation with TTF-1 immunohistochemistry EGFR mutational status. The lepidic predominant adenocarcinomas, papillary adenocarcinomas were associated with EGFR mutations and were significantly uncommon with invasive mucinous adenocarcinoma.

Limitations:

1. A major limitation of this study was that we did not include treatment strategies used and follow up cases. However, this limitation can be overcome to a certain extent using data that was recently published from our centre, where the overall survival (OS) and progression free survival (PFS) was assessed among 106 cases with a known EGFR mutational profile.
2. The subtype and the cell type have not been correlated with PFS and OS that would have to determine the association of the cell types from a prognostic standpoint.
3. EGFR mutational analysis by PCR gene sequencing was performed only in a subset of patients.
4. EGFR mutations were performed by Sanger's sequencing, which requires a minimum of 50% tumour cellularity, thereby limiting its utility among samples with lesser cellularity.

On a prospective basis, treatment protocols in these patients can be correlated with overall survival and progression free survival. We have now introduced more sensitive techniques like pyrosequencing for mutational analysis. We also aim to perform other mutation tests such as KRAS and EML4- ALK1 gene fusion for patients negative for EGFR mutations or not responding to treatment with TKI's.

Nevertheless, the study provides valuable information in the context of the histological patterns and the mutational status and can be used as baseline data for similar studies to perform on a larger scale.

SUMMARY &CONCLUSIONS

A detailed histopathological analysis of Primary Lung adenocarcinomas was done, using the new IASLC/ATS/ERS classification in biopsies and resection specimens from June 2011- December 2012 and this data was correlated with EGFR exon sequences performed in a subset using PCR gene sequencing.

The following are the results of this study summarized below:

1. The predominant categories of adenocarcinoma were, invasive acinar (61%), solid with mucin production (23%), invasive mucinous (19%), lepidic predominant (4%), papillary subtypes (3.30%) and the rare subtypes of colloid (0.7%), non small cell carcinoma- NOS (0.7%) and one case of micropapillary adenocarcinoma (0.3%). There were no cases of the fetal adenocarcinomas and adenocarcinomas with enteric differentiation.

2. The prevalence of EGFR mutations was 40.9%, mutations being present in one of the exons (18-21) detected by PCR gene sequencing (except one case that had a combined exon 20 and exon 21 mutation). The majority, 73.5% were associated with exon 19 mutations, with delE746-A750 gene mutation being the commonest in 69.4% cases. 24.5% cases were associated with exon 21 mutations, all of L858R gene type mutation.

3. EGFR mutations were seen mainly with lepidic predominant (60%) followedby papillary adenocarcinomas (44.4%), acinar adenocarcinomas (42.7%) and the solid subtype (41.7%) and were infrequent with the invasive mucinous subtype(P=0.015). The EGFR gene mutations were significantly associated with the cuboidal cells(P=0.013). EGFR mutations were relatively more common amongst women, non smokers and were significantly associated with TTF-1 positivity with a relative risk of 4.995.

4. Majority cases of Primary lung adenocarcinomas were TTF-1 positive (81%,P=0.007), mainly associated with lepidic and papillary subtypes.

5. On detailed histopathological analysis, the polygonal cell type was the commonest, frequently noted with the solid variant, stage IV disease and amongst smokers. The papillary subtype was associated with columnar cells.

6. A majority of tumours (55.1%) were poorly differentiated, 42% moderately differentiated and 2.9% were well differentiated. The solid predominant subtypes were commonly poorly differentiated (90.4%) and the papillary subtype was moderately differentiated (100%).

7. 69% of adenocarcinomas were associated with a lympho-plasmacytic response; however there was no correlation between inflammatory response and a particular subtype.

8. Majority were non smokers in this cohort and the solid variant was relatively frequent amongst smokers.

9. The right lung had a predilection for tumours (45.6%) and was bilateral.

10. Majority (86.4%) tumours on imaging were greater than 3 cm in greatest dimension and 82.2 % were in stage IV of disease.

Thus, in this study we performed a detailed histopathological study with clinico-pathological correlation and established a relationship between subtype, cell type and EGFR mutational analysis.

To our knowledge, in an Indian setting several studies have established a prevalence of EGFR mutations with NSCLC/adenocarcinomas though none have established a correlation between EGFR mutational status and lung adenocarcinoma in context of clinico-pathological features, predominant subtype and cell type using the 2011 IASLC/ATS/ERS classification.

Hence, we conclude that the new IASLC/ATS/ERS classification confirms the positive statistical association of the predominant subtype of invasive adenocarcinoma, cell type with EGFR mutations and TTF-1 reactivity.

Bibliography.

1. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA Cancer J Clin. 2011;61(2):69–90.

2. Ferlay J, Shin H-R, Bray F, Forman D, Mathers C, Parkin DM. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int J Cancer. 2010;127(12):2893–917.

3. Raparia K, Villa C, DeCamp MM, Patel JD, Mehta MP. Molecular profiling in non-small cell lung cancer: a step toward personalized medicine. Arch Pathol Lab Med. 2013 Apr;137(4):481–91.

4. Travis WD. Pathology & Genetics: Tumours of the Lung, Pleura, Thymus, and Heart [Internet]. Iarc; 2004 [cited 2013 Nov 15]. Available from: http://books.google.co.in/books?hl=en&lr=&id=pIbKfvQCtG4C&oi=fnd&pg=PA9&dq=Travis+WD,+Brambilla+E,+Konrad+Muller-Hermelink+H,+Harris+CC.+Pathology+and+Genetics+of+Tumours+of+the+Lung,+Pleura,+Thymus+and+Heart.+Lyon,+France:+IARC+Press%3B+2004.+World+Health+Organization+Classification+of+Tumours%3B+vol+10&ots=BLB6uMwYql&sig=W1vL0FYDcayzXsGjsWh5JWrAn_k

5. Motoi N, Szoke J, Riely GJ, Seshan VE, Kris MG, Rusch VW, et al. Lung adenocarcinoma: modification of the 2004 WHO mixed subtype to include the major histologic subtype suggests correlations between papillary and micropapillary adenocarcinoma subtypes, EGFR

mutations and gene expression analysis. Am J Surg Pathol. 2008;32(6):810.

6. Pikor LA, Ramnarine VR, Lam S, Lam WL. Genetic alterations defining NSCLC subtypes and their therapeutic implications. Lung Cancer Amst Neth. 2013 Nov;82(2):179–89.

7. Pesch B, Kendzia B, Gustavsson P, Jöckel K-H, Johnen G, Pohlabeln H, et al. Cigarette smoking and lung cancer—relative risk estimates for the major histological types from a pooled analysis of case–control studies. Int J Cancer. 2012;131(5):1210–9.

8. Kerr KM. Pulmonary adenocarcinomas: classification and reporting. Histopathology. 2009 Jan;54(1):12–27.

9. Gadgeel SM, Ramalingam S, Cummings G, Kraut MJ, Wozniak AJ, Gaspar LE, et al. Lung cancer in patients < 50 years of age: the experience of an academic multidisciplinary program. Chest. 1999 May;115(5):1232–6.

10. Itaya T, Yamaoto N, Ando M, Ebisawa M, Nakamura Y, Murakami H, et al. Influence of histological type, smoking history and chemotherapy on survival after first-line therapy in patients with advanced non-small cell lung cancer. Cancer Sci. 2007 Feb;98(2):226–30.

11. Colby TV, Koss M, Travis WD (1995). Tumors of the Lower Respiratory Tract. 3rded. Armed Forces Institute of Pathology:Washington, DC.

12. Travis WD, Brambilla E, Muller-Hermelink HK, et al. World Health Organization Classification of Tumours. Pathology and G.

13. Motoi N, Szoke J, Riely GJ, Seshan VE, Kris MG, Rusch VW, et al. Lung adenocarcinoma: modification of the 2004 WHO mixed subtype to include the major histologic subtype suggests correlations between papillary and micropapillary adenocarcinoma subtypes, EGFR mutations and gene expression analysis. Am J Surg Pathol. 2008 Jun;32(6):810–27.

14. Kerr KM. Pulmonary adenocarcinomas: classification and reporting. Histopathology. 2009 Jan;54(1):12–27.

15. Chilosi M, Murer B. Mixed adenocarcinomas of the lung: place in new proposals in classification, mandatory for target therapy. Arch Pathol Lab Med. 2010 Jan;134(1):55–65.

16. Amin MB, Tamboli P, Merchant SH, Ordóñez NG, Ro J, Ayala AG, et al. Micropapillary component in lung adenocarcinoma: a distinctive histologic feature with possible prognostic significance. Am J Surg Pathol. 2002 Mar;26(3):358–64.

17. Miyoshi T, Satoh Y, Okumura S, Nakagawa K, Shirakusa T, Tsuchiya E, et al. Early-stage lung adenocarcinomas with a micropapillary pattern, a distinct pathologic marker for a significantly poor prognosis. Am J Surg Pathol. 2003 Jan;27(1):101–9.

18. Travis WD, Brambilla E, Noguchi M, Nicholson AG, Geisinger K, Yatabe Y, et al. Diagnosis of Lung Adenocarcinoma in Resected

Specimens: Implications of the 2011 International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society Classification. Arch Pathol Lab Med. 2013 May;137(5):685–705.

19. Travis WD, Brambilla E, Noguchi M, Nicholson AG, Geisinger KR, Yatabe Y, et al. International association for the study of lung cancer/american thoracic society/european respiratory society international multidisciplinary classification of lung adenocarcinoma. J Thorac Oncol Off Publ Int Assoc Study Lung Cancer. 2011 Feb;6(2):244–85.

20. Travis WD, Brambilla E, Noguchi M, Nicholson AG, Geisinger K, Yatabe Y, et al. Diagnosis of lung cancer in small biopsies and cytology: implications of the 2011 International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society classification. Arch Pathol Lab Med. 2013 May;137(5):668–84.

21. Wang BY, Gil J, Kaufman D, Gan L, Kohtz DS, Burstein DE. P63 in pulmonary epithelium, pulmonary squamous neoplasms, and other pulmonary tumors. Hum Pathol. 2002 Sep;33(9):921–6.

22. Travis WD, Brambilla E, Noguchi M, Nicholson AG, Geisinger K, Yatabe Y, et al. International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society: international multidisciplinary classification of lung adenocarcinoma: executive summary. Proc Am Thorac Soc. 2011 Sep;8(5):381–5.

23. Okudela K, Woo T, Mitsui H, Yazawa T, Shimoyamada H, Tajiri M, et al. Proposal of an improved histological sub-typing system for lung adenocarcinoma–significant prognostic values for stage I disease. Int J Clin Exp Pathol. 2010;3(4):348.

24. Gaeta M, Blandino A, Scribano E, Ascenti G, Minutoli F, Pandolfo I. Mucinous cystadenocarcinoma of the lung: CT-pathologic correlation in three cases. J Comput Assist Tomogr. 1999 Aug;23(4):641–3.

25. Nakatani Y, Masudo K, Miyagi Y, Inayama Y, Kawano N, Tanaka Y, et al. Aberrant nuclear localization and gene mutation of beta-catenin in low-grade adenocarcinoma of fetal lung type: up-regulation of the Wnt signaling pathway may be a common denominator for the development of tumors that form morules. Mod Pathol Off J U S Can Acad Pathol Inc. 2002 Jun;15(6):617–24.

26. Mori M, Rao SK, Popper HH, Cagle PT, Fraire AE. Atypical adenomatous hyperplasia of the lung: a probable forerunner in the development of adenocarcinoma of the lung. Mod Pathol Off J U S Can Acad Pathol Inc. 2001 Feb;14(2):72–84.

27. Kitamura H, Kameda Y, Ito T, Hayashi H. Atypical adenomatous hyperplasia of the lung. Implications for the pathogenesis of peripheral lung adenocarcinoma. Am J Clin Pathol. 1999 May;111(5):610–22.

28. Westra WH. Early glandular neoplasia of the lung. Respir Res. 2000 Nov 17;1(3):163.

29. Soh J, Toyooka S, Ichihara S, Asano H, Kobayashi N, Suehisa H, et al. Sequential molecular changes during multistage pathogenesis of small peripheral adenocarcinomas of the lung. J Thorac Oncol Off Publ Int Assoc Study Lung Cancer. 2008 Apr;3(4):340–7.

30. Travis WD, Brambilla E, Noguchi M, Nicholson AG, Geisinger K, Yatabe Y, et al. International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society: international multidisciplinary classification of lung adenocarcinoma: executive summary. Proc Am Thorac Soc. 2011 Sep;8(5):381–5.

31. Klempner SJ, Cohen DW, Costa DB. ALK translocation in non-small cell lung cancer with adenocarcinoma and squamous cell carcinoma markers. J Thorac Oncol Off Publ Int Assoc Study Lung Cancer. 2011 Aug;6(8):1439–40.

32. Bishop JA, Teruya-Feldstein J, Westra WH, Pelosi G, Travis WD, Rekhtman N. p40 (ΔNp63) is superior to p63 for the diagnosis of pulmonary squamous cell carcinoma. Mod Pathol Off J U S Can Acad Pathol Inc. 2012 Mar;25(3):405–15.

33. Pelosi G, Fabbri A, Bianchi F, Maisonneuve P, Rossi G, Barbareschi M, et al. ΔNp63 (p40) and thyroid transcription factor-1 immunoreactivity on small biopsies or cellblocks for typing non-small cell lung cancer: a novel two-hit, sparing-material approach. J Thorac Oncol Off Publ Int Assoc Study Lung Cancer. 2012 Feb;7(2):281–90.

34. Sakurai H, Asamura H, Miyaoka E, Yoshino I, Fujii Y, Nakanishi Y, et al. Differences in the prognosis of resected lung adenocarcinoma according to the histological subtype: a retrospective analysis of Japanese lung cancer registry data. Eur J Cardio-Thorac Surg Off J Eur Assoc Cardio-Thorac Surg. 2014 Jan;45(1):100–7.

35. Yoshizawa A, Motoi N, Riely GJ, Sima CS, Gerald WL, Kris MG, et al. Impact of proposed IASLC/ATS/ERS classification of lung adenocarcinoma: prognostic subgroups and implications for further revision of staging based on analysis of 514 stage I cases. Mod Pathol Off J U S Can Acad Pathol Inc. 2011 May;24(5):653–64.

36. Yoshizawa A, Motoi N, Riely GJ, Sima CS, Gerald WL, Kris MG, et al. Impact of proposed IASLC/ATS/ERS classification of lung adenocarcinoma: prognostic subgroups and implications for further revision of staging based on analysis of 514 stage I cases. Mod Pathol Off J U S Can Acad Pathol Inc. 2011 May;24(5):653–64.

37. Anami Y, Iijima T, Suzuki K, Yokota J, Minami Y, Kobayashi H, et al. Bronchioloalveolar carcinoma (lepidic growth) component is a more useful prognostic factor than lymph node metastasis. J Thorac Oncol Off Publ Int Assoc Study Lung Cancer. 2009 Aug;4(8):951–8.

38. Sica G, Yoshizawa A, Sima CS, Azzoli CG, Downey RJ, Rusch VW, et al. A grading system of lung adenocarcinomas based on histologic pattern is predictive of disease recurrence in stage I tumors. Am J Surg Pathol. 2010 Aug;34(8):1155–62.

39. Ohtaki Y, Yoshida J, Ishii G, Aokage K, Hishida T, Nishimura M, et al. Prognostic Significance of a Solid Component in Pulmonary Adenocarcinoma. Ann Thorac Surg. 2011 Apr;91(4):1051–7.

40. Okada A, Shimmyo T, Hashimoto T, Kobayashi Y, Miyagi Y, Ishikawa Y, et al. Predictive advantage of a cell type classification for pulmonary adenocarcinoma coupled with data for p53, K-ras and EGFR alterations. Cancer Sci. 2010 Jul 1;101(7):1745–53.

41. Xu L, Tavora F, Battafarano R, Burke A. Adenocarcinomas with prominent lepidic spread: retrospective review applying new classification of the American Thoracic Society. Am J Surg Pathol. 2012 Feb;36(2):273–82.

42. Noguchi M, Morikawa A, Kawasaki M, Matsuno Y, Yamada T, Hirohashi S, et al. Small adenocarcinoma of the lung. Histologic characteristics and prognosis. Cancer. 1995 Jun 15;75(12):2844–52.

43. Tsutani Y, Miyata Y, Nakayama H, Okumura S, Adachi S, Yoshimura M, et al. Prognostic significance of using solid versus whole tumor size on high-resolution computed tomography for predicting pathologic malignant grade of tumors in clinical stage IA lung adenocarcinoma: A multicenter study. J Thorac Cardiovasc Surg. 2012;143(3):607–12.

44. Yoshizawa A, Motoi N, Riely GJ, Sima CS, Gerald WL, Kris MG, et al. Impact of proposed IASLC/ATS/ERS classification of lung adenocarcinoma: prognostic subgroups and implications for further

revision of staging based on analysis of 514 stage I cases. Mod Pathol. 2011;24(5):653–64.

45. Xu L, Tavora F, Battafarano R, Burke A. Adenocarcinomas with prominent lepidic spread: retrospective review applying new classification of the American Thoracic Society. Am J Surg Pathol. 2012 Feb;36(2):273–82.

46. Yoshizawa A, Motoi N, Riely GJ, Sima CS, Gerald WL, Kris MG, et al. Impact of proposed IASLC/ATS/ERS classification of lung adenocarcinoma: prognostic subgroups and implications for further revision of staging based on analysis of 514 stage I cases. Mod Pathol Off J U S Can Acad Pathol Inc. 2011 May;24(5):653–64.

47. Engels EA. Inflammation in the development of lung cancer: epidemiological evidence. Expert Rev Anticancer Ther. 2008 Apr;8(4):605–15.

48. Ballaz S, Mulshine JL. The potential contributions of chronic inflammation to lung carcinogenesis. Clin Lung Cancer. 2003 Jul;5(1):46–62.

49. Perwez Hussain S, Harris CC. Inflammation and cancer: An ancient link with novel potentials. Int J Cancer. 2007 Dec 1;121(11):2373–80.

50. Russell PA, Wainer Z, Wright GM, Daniels M, Conron M, Williams RA. Does lung adenocarcinoma subtype predict patient survival?: A clinicopathologic study based on the new International Association for the Study of Lung Cancer/American Thoracic Society/European

Respiratory Society international multidisciplinary lung adenocarcinoma classification. J Thorac Oncol. 2011;6(9):1496–504.

51. Yoshizawa A, Sumiyoshi S, Sonobe M, Kobayashi M, Fujimoto M, Kawakami F, et al. Validation of the IASLC/ATS/ERS lung adenocarcinoma classification for prognosis and association with EGFR and KRAS gene mutations: analysis of 440 Japanese patients. J Thorac Oncol Off Publ Int Assoc Study Lung Cancer. 2013 Jan;8(1):52–61.

52. Ye J, Hameed O, Findeis-Hosey JJ, Fan L, Li F, McMahon LA, et al. Diagnostic utility of PAX8, TTF-1 and napsin A for discriminating metastatic carcinoma from primary adenocarcinoma of the lung. Biotech Histochem Off Publ Biol Stain Comm. 2012 Jan;87(1):30–4.

53. Kim M-Y, Go H, Koh J, Lee K, Min H-S, Kim M-A, et al. Napsin A is a useful marker for metastatic adenocarcinomas of pulmonary origin. Histopathology. 2014 Aug;65(2):195–206.

54. Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, Brannigan BW, et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. N Engl J Med. 2004 May 20;350(21):2129–39.

55. Noronha V, Dikshit R, Raut N, Joshi A, Pramesh CS, George K, et al. Epidemiology of lung cancer in India: focus on the differences between non-smokers and smokers: a single-centre experience. Indian J Cancer. 2012 Mar;49(1):74–81.

56. Bethune G, Bethune D, Ridgway N, Xu Z. Epidermal growth factor receptor (EGFR) in lung cancer: an overview and update. J Thorac Dis. 2010 Mar;2(1):48–51.

57. Inamura K, Ninomiya H, Ishikawa Y, Matsubara O. Is the epidermal growth factor receptor status in lung cancers reflected in clinicopathologic features? Arch Pathol Lab Med. 2010 Jan;134(1):66–72.

58. Bethune G, Bethune D, Ridgway N, Xu Z. Epidermal growth factor receptor (EGFR) in lung cancer: an overview and update. J Thorac Dis. 2010 Mar;2(1):48–51.

59. Matsumoto S, Takahashi K, Iwakawa R, Matsuno Y, Nakanishi Y, Kohno T, et al. Frequent EGFR mutations in brain metastases of lung adenocarcinoma. Int J Cancer. 2006;119(6):1491–4.

60. Inamura K, Ninomiya H, Ishikawa Y, Matsubara O. Is the epidermal growth factor receptor status in lung cancers reflected in clinicopathologic features? Arch Pathol Lab Med. 2010 Jan;134(1):66–72.

61. Rosell R, Moran T, Queralt C, Porta R, Cardenal F, Camps C, et al. Screening for epidermal growth factor receptor mutations in lung cancer. N Engl J Med. 2009 Sep 3;361(10):958–67.

62. Pao W, Miller V, Zakowski M, Doherty J, Politi K, Sarkaria I, et al. EGF receptor gene mutations are common in lung cancers from “never

smokers” and are associated with sensitivity of tumors to gefitinib and erlotinib. Proc Natl Acad Sci U S A. 2004 Sep 7;101(36):13306–11.

63. Massarelli E, Varella-Garcia M, Tang X, Xavier AC, Ozburn NC, Liu DD, et al. KRAS mutation is an important predictor of resistance to therapy with epidermal growth factor receptor tyrosine kinase inhibitors in non-small-cell lung cancer. Clin Cancer Res Off J Am Assoc Cancer Res. 2007 May 15;13(10):2890–6.

64. Amann J, Kalyankrishna S, Massion PP, Ohm JE, Girard L, Shigematsu H, et al. Aberrant epidermal growth factor receptor signaling and enhanced sensitivity to EGFR inhibitors in lung cancer. Cancer Res. 2005 Jan 1;65(1):226–35.

65. Li AR, Chitale D, Riely GJ, Pao W, Miller VA, Zakowski MF, et al. EGFR Mutations in Lung Adenocarcinomas. J Mol Diagn JMD. 2008 May;10(3):242–8.

66. González Manzano R, Martínez Navarro E, Eugenieva E, Fernández Morejón FJ, Farré J, Brugarolas A. A novel EGFR nonsense mutation in a non-small-cell lung cancer (NSCLC) patient who did not derive any clinical benefit with combination chemotherapy and erlotinib. Clin Transl Oncol Off Publ Fed Span Oncol Soc Natl Cancer Inst Mex. 2008 Jul;10(7):442–4.

67. Jackman DM, Yeap BY, Sequist LV, Lindeman N, Holmes AJ, Joshi VA, et al. Exon 19 deletion mutations of epidermal growth factor receptor are associated with prolonged survival in non–small cell lung

cancer patients treated with gefitinib or erlotinib. Clin Cancer Res. 2006;12(13):3908–14.

68. Cohen V, Agulnik JS, Ang C, Kasymjanova G, Batist G, Small D, et al. Epidermal growth factor receptor mutations detected by denaturing high-performance liquid chromatography in nonsmall cell lung cancer. Cancer. 2010 Sep 15;116(18):4309–17.

69. Jang TW, Oak CH, Chang HK, Suo SJ, Jung MH. EGFR and KRAS mutations in patients with adenocarcinoma of the lung. Korean J Intern Med. 2009 Mar;24(1):48–54.

70. Bae NC, Chae MH, Lee MH, Kim KM, Lee EB, Kim CH, et al. EGFR, ERBB2, and KRAS mutations in Korean non-small cell lung cancer patients. Cancer Genet Cytogenet. 2007 Mar;173(2):107–13.

71. Gao B, Sun Y, Zhang J, Ren Y, Fang R, Han X, et al. Spectrum of LKB1, EGFR, and KRAS mutations in chinese lung adenocarcinomas. J Thorac Oncol Off Publ Int Assoc Study Lung Cancer. 2010 Aug;5(8):1130–5.

72. Sequist LV, Martins RG, Spigel D, Grunberg SM, Spira A, Janne PA, et al. First-Line Gefitinib in Patients With Advanced Non-Small-Cell Lung Cancer Harboring Somatic EGFR Mutations. J Clin Oncol. 2008 May 20;26(15):2442–9.

73. Bhatt AD, Pai R, Rebekah G, Nehru GA, Dhananjayan S, Samuel A, et al. Clinicopathologic features of non-small cell lung cancer in India and

correlation with epidermal growth factor receptor mutational status. Indian J Cancer. 2013 Jun;50(2):94–101.

74. Doval DC, Azam S, Batra U, Choudhury KD, Talwar V, Gupta SK, et al. Epidermal growth factor receptor mutation in lung adenocarcinoma in India: A single center study. J Carcinog. 2013;12(1):12.

75. Sahoo R, Harini VV, Babu VC, Patil Okaly GV, Rao S, Nargund A, et al. Screening for EGFR mutations in lung cancer, a report from India. Lung Cancer Amst Neth. 2011 Sep;73(3):316–9.

76. Sun P-L, Seol H, Lee HJ, Yoo SB, Kim H, Xu X, et al. High incidence of EGFR mutations in Korean men smokers with no intratumoral heterogeneity of lung adenocarcinomas: correlation with histologic subtypes, EGFR/TTF-1 expressions, and clinical features. J Thorac Oncol Off Publ Int Assoc Study Lung Cancer. 2012 Feb;7(2):323–30.

77. Vallee A, Sagan C, Le Loupp A-G, Bach K, Dejoie T, Denis MG. Detection of EGFR gene mutations in non-small cell lung cancer: lessons from a single-institution routine analysis of 1,403 tumor samples. Int J Oncol. 2013 Oct;43(4):1045–51.

78. Yoshizawa A, Sumiyoshi S, Sonobe M, Kobayashi M, Fujimoto M, Kawakami F, et al. Validation of the IASLC/ATS/ERS lung adenocarcinoma classification for prognosis and association with EGFR and KRAS gene mutations: analysis of 440 Japanese patients. J Thorac Oncol Off Publ Int Assoc Study Lung Cancer. 2013 Jan;8(1):52–61.

79. Sun P-L, Seol H, Lee HJ, Yoo SB, Kim H, Xu X, et al. High incidence of EGFR mutations in Korean men smokers with no intratumoral heterogeneity of lung adenocarcinomas: correlation with histologic subtypes, EGFR/TTF-1 expressions, and clinical features. J Thorac Oncol Off Publ Int Assoc Study Lung Cancer. 2012 Feb;7(2):323–30.

80. Garfield DH, Cadranel J, West HL. Bronchioloalveolar carcinoma: the case for two diseases. Clin Lung Cancer. 2008 Jan;9(1):24–9.

81. Finberg KE, Sequist LV, Joshi VA, Muzikansky A, Miller JM, Han M, et al. Mucinous differentiation correlates with absence of EGFR mutation and presence of KRAS mutation in lung adenocarcinomas with bronchioloalveolar features. J Mol Diagn JMD. 2007 Jul;9(3):320–6.

82. Sakuma Y, Matsukuma S, Yoshihara M, Nakamura Y, Noda K, Nakayama H, et al. Distinctive evaluation of nonmucinous and mucinous subtypes of bronchioloalveolar carcinomas in EGFR and K-ras gene-mutation analyses for japanese lung adenocarcinomas confirmation of the correlations with histologic subtypes and gene mutations. Am J Clin Pathol. 2007;128(1):100–8.

83. Nakano H, Soda H, Takasu M, Tomonaga N, Yamaguchi H, Nakatomi K, et al. Heterogeneity of epidermal growth factor receptor mutations within a mixed adenocarcinoma lung nodule. Lung Cancer Amst Neth. 2008 Apr;60(1):136–40.

84. Sakurada A, Lara-Guerra H, Liu N, Shepherd FA, Tsao M-S. Tissue heterogeneity of EGFR mutation in lung adenocarcinoma. J Thorac Oncol Off Publ Int Assoc Study Lung Cancer. 2008 May;3(5):527–9.

85. Yatabe Y, Matsuo K, Mitsudomi T. Heterogeneous distribution of EGFR mutations is extremely rare in lung adenocarcinoma. J Clin Oncol Off J Am Soc Clin Oncol. 2011 Aug 1;29(22):2972–7.

86. Kosaka T, Yatabe Y, Onozato R, Kuwano H, Mitsudomi T. Prognostic implication of EGFR, KRAS, and TP53 gene mutations in a large cohort of Japanese patients with surgically treated lung adenocarcinoma. J Thorac Oncol Off Publ Int Assoc Study Lung Cancer. 2009 Jan;4(1):22–9.

87. Marks JL, Broderick S, Zhou Q, Chitale D, Li AR, Zakowski MF, et al. Prognostic and therapeutic implications of EGFR and KRAS mutations in resected lung adenocarcinoma. J Thorac Oncol Off Publ Int Assoc Study Lung Cancer. 2008 Feb;3(2):111–6.

88. Lindeman NI, Cagle PT, Beasley MB, Chitale DA, Dacic S, Giaccone G, et al. Molecular testing guideline for selection of lung cancer patients for EGFR and ALK tyrosine kinase inhibitors: guideline from the College of American Pathologists, International Association for the Study of Lung Cancer, and Association for Molecular Pathology. J Mol Diagn JMD. 2013 Jul;15(4):415–53.

89. Oxnard GR, Arcila ME, Sima CS, Riely GJ, Chmielecki J, Kris MG, et al. Acquired resistance to EGFR tyrosine kinase inhibitors in EGFR-

mutant lung cancer: distinct natural history of patients with tumors harboring the T790M mutation. Clin Cancer Res Off J Am Assoc Cancer Res. 2011 Mar 15;17(6):1616–22.

90. Bean J, Brennan C, Shih J-Y, Riely G, Viale A, Wang L, et al. MET amplification occurs with or without T790M mutations in EGFR mutant lung tumors with acquired resistance to gefitinib or erlotinib. Proc Natl Acad Sci. 2007;104(52):20932–7.

91. Kosaka T, Yatabe Y, Endoh H, Yoshida K, Hida T, Tsuboi M, et al. Analysis of epidermal growth factor receptor gene mutation in patients with non-small cell lung cancer and acquired resistance to gefitinib. Clin Cancer Res Off J Am Assoc Cancer Res. 2006 Oct 1;12(19):5764–9.

92. Engelman JA, Zejnullahu K, Mitsudomi T, Song Y, Hyland C, Park JO, et al. MET amplification leads to gefitinib resistance in lung cancer by activating ERBB3 signaling. Science. 2007;316(5827):1039–43.

93. Shibata T, Uryu S, Kokubu A, Hosoda F, Ohki M, Sakiyama T, et al. Genetic classification of lung adenocarcinoma based on array-based comparative genomic hybridization analysis: its association with clinicopathologic features. Clin Cancer Res Off J Am Assoc Cancer Res. 2005 Sep 1;11(17):6177–85.

94. Kosaka T, Yatabe Y, Endoh H, Kuwano H, Takahashi T, Mitsudomi T. Mutations of the epidermal growth factor receptor gene in lung cancer:

biological and clinical implications. Cancer Res. 2004 Dec 15;64(24):8919–23.

95. Inoue A, Suzuki T, Fukuhara T, Maemondo M, Kimura Y, Morikawa N, et al. Prospective phase II study of gefitinib for chemotherapy-naive patients with advanced non-small-cell lung cancer with epidermal growth factor receptor gene mutations. J Clin Oncol Off J Am Soc Clin Oncol. 2006 Jul 20;24(21):3340–6.

96. Choi YL, Soda M, Yamashita Y, Ueno T, Takashima J, Nakajima T, et al. EML4-ALK mutations in lung cancer that confer resistance to ALK inhibitors. N Engl J Med. 2010 Oct 28;363(18):1734–9.

97. Shen S, Qin D. Pyrosequencing data analysis software: a useful tool for EGFR, KRAS, and BRAF mutation analysis. Diagn Pathol. 2012;7(1):56.

98. Ha SY, Roh MS. The new 2011 international association for the study of lung cancer/american thoracic society/european respiratory society classification of lung adenocarcinoma in resected specimens: clinicopathologic relevance and emerging issues. Korean J Pathol. 2013 Aug;47(4):316–25.

99. Veldore VH, Rao RM, Kakara S, Pattanayak S, Tejaswi R, Sahoo R, et al. Epidermal growth factor receptor mutation in non-small-cell lung carcinomas: a retrospective analysis of 1036 lung cancer specimens from a network of tertiary cancer care centers in India. Indian J Cancer. 2013 Jun;50(2):87–93.

100. Mehta J. Molecular epidemiology of epidermal growth factor receptor mutations in lung cancers in Indian population. Indian J Cancer. 2013 Jun;50(2):102–6.

101. Noronha V, Prabhash K, Thavamani A, Chougule A, Purandare N, Joshi A, et al. EGFR mutations in Indian lung cancer patients: clinical correlation and outcome to EGFR targeted therapy. PloS One. 2013;8(4):e61561.

102. Sun P-L, Seol H, Lee HJ, Yoo SB, Kim H, Xu X, et al. High incidence of EGFR mutations in Korean men smokers with no intratumoral heterogeneity of lung adenocarcinomas: correlation with histologic subtypes, EGFR/TTF-1 expressions, and clinical features. J Thorac Oncol Off Publ Int Assoc Study Lung Cancer. 2012 Feb;7(2):323–30.

103. Okada A, Shimmyo T, Hashimoto T, Kobayashi Y, Miyagi Y, Ishikawa Y, et al. Predictive advantage of a cell type classification for pulmonary adenocarcinoma coupled with data for p53, K-ras and EGFR alterations. Cancer Sci. 2010 Jul 1;101(7):1745–53.

PROFORMA

1. Serial no: 2. Biopsy no: 3.Age: 4. Gender : M/F

5. Geog location: North/West/East/South India/others

6.Smoking: Smoker/Non smoker .

Radiological Findings :

7.Tumour laterality: Right /Left/Bilateral **8. Primary lobe**: Upper/middle lobe Or lower lobe.

9.Maximum tumour : <3.0cm/ >3.0cm. **10. Associated lymphadenopathy**: Yes/ No.

11. Stage of disease: Gross findings (resection specimens)

12.Tumour size: < 3cm/>3cm. **13.Site**: Upper/middle lobe or lower lobe.

14. Circumscription: Absent/ Present.**15.Infiltrative margins**: Absent/ present.

16.Pleural involvement: Absent/ present 1**7.Haemorrhage** : Absent/ present.

18. Necrosis: Absent/ present.

Histopathological subtypes:

19.BAC : Absent/present. If present, then Invasive lepidic predominent/AIS/MIA

20. **Papillary** : Absent/ present. **21**.**Micropapillary**: Absent/ Present**.**

22. Acinar : Absent/Present. **23.Solid component**: Absent/ Present.

24. **Colloid:** Absent/Present. **25**. **Mucinous:** Absent/Present.

26. NSCLC-NOS: Absent/Present.

Microscopic details.

27.Predominant cell type: Columnar/ Cuboidal/ Hobnail/Clear cell/Polygonal/Signet.

28.Associated with mucin : Yes/ No. **29**.**Desmoplasia :** Absent/ Present.

30.Stromal elastosis : Absent/Present**.** **31.Lymphatic permeation** :Absent /Present.

32. Inflammatory cell infiltrate Acute / Chronic/ Mixed.

33. Necrosis: Absent/ Present. **34**. **Tumour grade**: Well/ Moderate/Poorly differentiated.

35. TTF-1 Positive/Negative. **36**. **CK7**Positive/Negative.

37. **CK20**: Positive/Negative. **38.BerEp4-** Negative/ Positive.

39.**EGFR mutational analysis**: Wild type/ Mutated If mutated which exon was amplified.

40.**Gene mutation associated**.

INSTITUTIONAL REVIEW BOARD (IRB)
CHRISTIAN MEDICAL COLLEGE
VELLORE 632 002, INDIA

Dr.B.J.Prashantham, M.A.,M.A.,Dr.Min(Clinical)
Director, Christian Counseling Centre
Editor, Indian Journal of Psychological Counseling
Chairperson, Ethics Committee, IRB

Dr. Alfred Job Daniel, MS Ortho
Chairperson, Research Committee &
Principal

Dr. Nihal Thomas
MD, MNAMS, DNB(Endo), FRACP(Endo), FRCP(Edin)
Secretary, Ethics Committee, IRB
Additional Vice Principal (Research)

January 4, 2013

Dr. Ravi Priyanka Yogendra
Department of General Pathology
Christian Medical College
Vellore 632 002

Sub: **FLUID Research grant project NEW PROPOSAL:**
Histopathologic features with epidermal growth factor receptor mutational analysis by polymerase chain reaction of primary lung adenocarcinomas including poorly differentiated carcinomas.
Dr. Ravi Priyanka Yogendra, General Pathology, Dr. Anila Korula, General pathology, Dr. Rekha Pai, Pathology, Dr. Visaklakshi J, Biostatistics

Ref: IRB Min. No. 8065 dated 06.11.2012

Dear Dr. Ravi Priyanka Yogendra,

I enclose the following documents:-

1. Institutional Review Board approval
2. Agreement

Could you please sign the agreement and send it to Dr. Nihal Thomas, Addl. Vice Principal (Research), so that the grant money can be released.

With best wishes,

Dr. Nihal Thomas
Secretary (Ethics Committee)
Institutional Review Board

Dr Nihal Thomas
MBBS MD MNAMS DNB (Endo) FRACP(Endo) FRCP(Edin)
Secretary (Ethics Committee)
Institutional Review Board

CC: Dr. Anila Korula, Department of General Pathology

INSTITUTIONAL REVIEW BOARD (IRB)
CHRISTIAN MEDICAL COLLEGE
VELLORE 632 002, INDIA

Dr.B.J.Prashantham, M.A.,M.A.,Dr.Min(Clinical)
Director, Christian Counseling Centre
Editor, Indian Journal of Psychological Counseling
Chairperson, Ethics Committee, IRB

Dr. Alfred Job Daniel, MS Ortho
Chairperson, Research Committee &
Principal

Dr. Nihal Thomas
MD, MNAMS, DNB(Endo), FRACP(Endo), FRCP(Edin)
Secretary, Ethics Committee, IRB
Additional Vice Principal (Research)

January 4, 2013

Dr. Ravi Priyanka Yogendra
Department of General Pathology
Christian Medical College
Vellore 632 002

Sub: **FLUID Research grant project NEW PROPOSAL:**
Histopathologic features with epidermal growth factor receptor mutational analysis by polymerase chain reaction of primary lung adenocarcinomas including poorly differentiated carcinomas.
Dr. Ravi Priyanka Yogendra, General Pathology, Dr. Anila Korula, General pathology, Dr. Rekha Pai, Pathology, Dr. Visaklakshi J, Biostatistics

Ref: IRB Min. No. 8065 dated 06.11.2012

Dear Dr. Ravi Priyanka Yogendra,

The Institutional Review Board (Blue, Research and Ethics Committee) of the Christian Medical College, Vellore, reviewed and discussed your project entitled "Histopathologic features with epidermal growth factor receptor mutational analysis by polymerase chain reaction of primary lung adenocarcinomas including poorly differentiated carcinomas." on November 6, 2012.

The Committees reviewed the following documents:

1. Format for application to IRB submission
2. Proforma
3. Cvs of Drs. Ravi Priyanka Yogendra, Anila Korula, Rekha Pai, Visalakshi.
4. A CD containing documents 1 – 3

The following Institutional Review Board (Research & Ethics Committee) members were present at the meeting held on November 6, 2012 in the CREST/SACN Conference Room, Christian Medical College, Bagayam, Vellore 632002.

INSTITUTIONAL REVIEW BOARD (IRB)
CHRISTIAN MEDICAL COLLEGE
VELLORE 632 002, INDIA

Dr.B.J.Prashantham, M.A.,M.A.,Dr.Min(Clinical)
Director, Christian Counseling Centre
Editor, Indian Journal of Psychological Counseling
Chairperson, Ethics Committee, IRB

Dr. Alfred Job Daniel, MS Ortho
Chairperson, Research Committee &
Principal

Dr. Nihal Thomas
MD, MNAMS, DNB(Endo), FRACP(Endo), FRCP(Edin)
Secretary, Ethics Committee, IRB
Additional Vice Principal (Research)

Dr. Nihal Thomas	MD MNAMS DNB(Endo) FRACP(Endo) FRCP(Edin)	Secretary IRB (EC)& Dy. Chairperson (IRB), Professor of Endocrinology & Addl. Vice Principal (Research), CMC.	Internal, Clinician

We approve the project to be conducted as presented.

The Institutional Ethics Committee expects to be informed about the progress of the project, any serious adverse events occurring in the course of the project, any changes in the protocol and the patient information/informed consent. And on completion of the study you are expected to submit a copy of the final report.

A sum of Rs 56,800/- (Rupees Fifty Six Thousand Eight Hundred only) will be granted for one and half years.

Yours sincerely,

Dr. Nihal Thomas
Secretary (Ethics Committee)
Institutional Review Board

Dr Nihal Thomas
MBBS MD MNAMS DNB (Endo) FRACP(Endo) FRCP(Edin)
Secretary (Ethics Committee)
Institutional Review Board

CC: Dr. Anila Korula, Department of General Pathology